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NOVARTIS

October 22, 1997

Executive Secretary
Dr. Larry G. Hart
P. O. Box 12233
Research Triangle Park, NC 27709

RE: 9th Report on Carcinogens - Phenolphthalein Data

Dear Dr. Hart:

This submission comprises the materials to be presented by Novartis Consumer Health, Inc., (NCHI) at the public meeting on October 30 & 31, 1997, and additional background information. This submission focuses on phenolphthalein.

Recent studies conducted by the National Toxicology Program have been interpreted to mean that, based on findings of rodent carcinogenicity and genotoxicity in several test systems, phenolphthalein was a potential human carcinogen. NCHI respectfully disagrees with this conclusion. Examination of the same data by other recognized experts does not support the conclusion of a genotoxic mechanism for phenolphthalein. The reasons for this position are detailed in the annexed reports from independent consulting toxicologist Dr. Francis Roe and at CanTox, Inc., which are made part of this submission.

Based on a review of the totality of the data base on phenolphthalein, NCHI continues to believe that many important questions concerning phenolphthalein and the relevance of the experimental data remain to be answered. In considering the weight of the scientific evidence, the hypothesis that phenolphthalein acts through a genotoxic mechanism has not been proven. On the contrary, there is clear evidence for aneugenic properties, which help to explain some of the high-dose tumor effects since aneuploidy-inducing chemicals are generally assumed to have thresholds of action.

Furthermore, while the transgenic p53 mouse may prove to be a valuable research tool once it is more fully understood, for the current data to be used as a basis for classifying phenolphthalein as a genotoxic carcinogen would set an unfortunate scientific precedent. Inconsistencies between the results of the genetically disabled p53 mouse assay on phenolphthalein and the two year carcinogenicity studies, as well as questions about the potential mechanism of action of phenolphthalein as a genotoxic carcinogen, call into question the usefulness of the p53 model for this particular compound.

The dosage question is another point to consider in the interpretation of the results from the experimental p53 transgenic system. The application of exposure data from tumor suppressor

deficient rodents in quantitative human risk assessment will require further investigation. The p53 mouse model is designed to be sensitive, and whatever mechanistic relevance it may achieve with additional research may prove to be extremely valuable in a qualitative manner. However, to use these test results quantitatively for extrapolation to the human population is not supported at this time.

In conclusion, there remains no relevant human data that calls into question the safety of phenolphthalein, and full consideration of all the available data on this compound continue to support its status as "generally recognized as safe."

Respectfully submitted,

Novartis Consumer Health, Inc.

FOR

Mark Gelbert, Ph.D., J.D.
Vice President
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Enclosure

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EVALUATION OF THE RODENT CARCINOGENICITY OF PHENOLPHTHALEIN

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EXECUTIVE SUMMARY

Chronic dietary bioassays on phenolphthalein showed increased incidences of adrenal medullary pheochromocytomas in treated F344 rats, renal tubular cell tumors in male rats, histiocytic sarcomas and thymic lymphomas in treated B6C3F$_1$ mice and benign ovarian tumors in treated female mice (NTP, 1996). In a subsequent study, using the alternative *p53+/-* transgenic mouse model to investigate the mechanism of tumor induction in female mice, thymic lymphomas were found (Tice and Furedi-Machacek, 1997). The potential relevance to humans of these tumors, and possible mechanisms involved in their development, are the subject of this review.

There are large differences, in duration and exposure between the lifetime rodent dietary exposures in the NTP (1996) study (starting at age 6 to 7 weeks) and the adult doses potentially received by humans. Humans generally receive phenolphthalein on an intermittent basis, as a result of the use of phenolphthalein as a laxative. Increased incidences of certain tumor types in rodents linked to high-dose toxicity, or other non-genotoxic mechanisms that are operative only at high-doses, would have no relevance to humans exposed at therapeutic doses.

On the basis of this review, the rat adrenal medullary pheochromocytomas are concluded to have been the likely result of a rat-specific mechanism of action. Also, these tumors are considered of no relevance to humans due to the known differences between rats and humans in the incidence of spontaneous and induced adrenal medullary lesions and in the morphological and functional properties of the lesions themselves. The renal tumors in phenolphthalein-treated male rats are concluded to be the result of high-dose toxicity, including the exacerbation of chronic progressive nephropathy, a condition in the advanced stages which is known to lead to an increased incidence of renal tubular cell neoplasms (Hard, 1990; Montgomery and Seely, 1990; Alden and Frith, 1991). Therefore, the tumors observed in the rat are entirely consistent with non-genotoxic mechanisms which have no relevance to human exposure at therapeutic doses.

With respect to the increased incidence of tumors reported in phenolphthalein-treated mice, there is sufficient evidence to conclude that they also were not the result of a genotoxic mechanism. First, with respect to the ovarian tumors, the histopathology data indicate a clear effect of phenolphthalein on the hormonal status of the female mice as well as evidence of tumor development *via* a hormonal mechanism (*i.e.*, observation of hyperplasia and benign tumors only). The demonstrated estrogenic activity of phenolphthalein and the absence of ovarian tumors in the subsequent *p53*-deficient mouse study (Tice and Furedi-Machacek, 1997) further demonstrate that these tumors did not arise by a genotoxic mechanism, but instead required long-term exposure to a weakly estrogenic substance.

Second, for the histiocytic sarcomas, the histological data showed evidence of high-dose toxicity (*i.e.*, myelofibrosis, pigmentation, bone marrow hypoplasia, hematopoietic cell proliferation in the red pulp of the spleen) and of estrogenic activity. Estrogens are known to produce toxic effects in the hematopoietic system of mice and to induce tumors of the hematopoietic system in this species (reviewed in IARC, 1979). These observations indicate that the histiocytic sarcomas in mice in the NTP study likely developed as the result of this non-genotoxic mechanism of action. The lack of induction of histiocytic sarcomas in the *p53*+/- assay (Tice and Furedi-Machacek, 1997) further supports this conclusion.

Finally, as with the histiocytic sarcomas, based on the results of carcinogenicity studies with estrogens, it is likely that the weakly estrogenic activity of phenolphthalein played a role in the development of the thymic lymphomas in the NTP (1996) study, and the single tumor type to be confirmed in the *p53*+/- assay (Tice and Furedi-Machacek, 1997). Evidence indicating altered hormonal status was seen in the observation of decreased incidences of proliferative lesions of the pituitary gland, thyroid gland, and liver. In any case, the mechanism likely involved in the development of the thymic lymphomas is threshold-dependent (*i.e.*, receptor mediated or expressed only at high-doses), with negligible carcinogenic risk to humans at therapeutic doses. The histopathological data and the results of various genotoxicity tests and of the *p53*+/- transgenic mouse assay, demonstrate an aneugenic mechanism to be involved in the development of the thymic lymphomas reported to occur in the NTP (1996) bioassay and in the later alternative model study (Tice and Furedi-Machacek, 1997). Further consideration of the micronucleus data and the chromosomal loss analysis in lymphoma cells, coupled with the absence of DNA damage in the very sensitive single cell gel assay, indicates that thymic

lymphomas in the *p53+/-* test arose through an aneugenic mechanism. Aneugenic mechanisms are threshold-dependent and, as such, remove phenolphthalein from inclusion in the category of genotoxic non-threshold rodent carcinogens. This is an important point with respect to the extrapolation of potential risks from rodents to humans.

Finally, it is important to comment on the use of the experimental data generated from the *p53+/-* transgenic mouse as an alternative model under evaluation. There is great hope that this system will prove to be of valuable use in regulatory decision making. It is premature, first of all, to attempt to extrapolate quantitatively, from dose levels used to treat the genetically-disabled and sensitized *p53+/-* mice, to human exposure. These research studies by Tice and Furedi-Machacek (1997) and Dunnick *et al.* (1997) were very well done, and represent a comprehensive attempt to understand mechanism of action of a rodent carcinogen. It is worrisome, however, that they have been interpreted to be evidence of phenolphthalein genotoxicity since they show clearly that:

1) the hypothesis is unproven that the ovarian tumors found in the NTP (1996) assay arose through a genotoxic mechanism;
2) there is a clear absence of DNA damage in the single cell gel electrophoresis (SCGE) assay, which is exquisitely sensitive in detecting genotoxicity;
3) a preponderance of micronuclei induced in *p53+/-* mice contained kinetochore material and, thus, likely arose through a threshold mechanism of aneugenesis rather than the non-threshold mechanism of genotoxicity; and,
4) loss of heterozygosity of the *p53* gene in 100% of the thymic lymphomas analyzed shows clear evidence of an aneugenic mechanism rather than genotoxicity.

In the face of this overwhelming evidence for non-genotoxicity in the *p53+/-* system and related tests, for any regulatory agency to draw a conclusion of genotoxicity from this evidence and base a regulatory decision on this conclusion, would set a most unfortunate precedent.

In conclusion, non-genotoxic mechanisms account for the tumors observed in the bioassays conducted with phenolphthalein (NTP, 1996; Tice and Furedi-Machacek, 1997). The pathological evidence, which points to non-genotoxic modes of action, and the

pharmacokinetic data, demonstrating that the animals in the NTP (1996) study experienced plasma concentrations of phenolphthalein which were up to 100-fold greater than potentially attainable in adults exposed to therapeutic doses, provide convincing evidence to indicate that phenolphthalein presents no carcinogenic risk to humans at therapeutic doses.

EVALUATION OF THE RODENT CARCINOGENICITY OF PHENOLPHTHALEIN

1.0 GENERAL INTRODUCTION

The Food and Drug Administration (FDA) announced a reopening of the administrative record and a proposal to amend the tentative final monograph for over-the-counter (OTC) laxative drug products (Federal Register 62(16):4622-46227, September 2, 1997). In this notice, FDA is proposing to reclassify phenolphthalein from Category I (generally recognized as safe and effective and not misbranded) to Category II (not generally recognized as safe and effective or misbranded) and to add this compound to a list of non-monograph ingredients. This action by FDA follows the statement that the Agency considers the use of phenolphthalein a potential carcinogenic risk to humans, based upon new data from an alternative model under evaluation, which reportedly support a genotoxic mechanism of tumorigenesis in rodent carcinogenicity bioassays.

At the request of Novartis Consumer Health, CanTox was asked to provide a critical review, evaluation, and interpretation of the database that led to FDA's recommendation. This assessment which follows leads to the conclusion that rodent tumorigenicity of phenolphthalein is mediated through non-genotoxic mechanisms, a point of critical importance to the determination of the potential risks to laxative users. This review references some of the points and discussion submitted previously (CanTox, 1997; attached as Appendix A).

Most of the discussion that has taken place has focused on risk in the absence of any comments on the benefits derived from phenolphthalein. A number of significant reasons support the ongoing availability of stimulant laxative OTC drug products. Stimulant laxatives are a valuable option to other types of laxative products in the treatment of constipation. The use of stimulant laxatives is especially important for refractory cases not rectified by other laxative agents, including bulk-formers and hyperosmotic agents. The stimulant laxatives may also be preferred alternatives to bulk-formers or lactulose-containing laxatives due to undesirable side effects such as gas and bloating in some individuals. In addition, the oral dosage form of

stimulant laxatives produces bowel movement in a significantly shorter period of time than some other active ingredients, especially the bulk-forming products and lubricants.

This review is organized chronologically to the extent that it begins with a discussion of the National Toxicology Program (NTP) chronic bioassays in rats and mice (NTP, 1996). It also includes the more recent "cutting-edge" research by NTP (Tice and Furedi-Machacek, 1997) conducted to investigate the possibility of a genotoxic mechanism to explain the bioassay results. This research involved an alternative model system, the *p53+/-* transgenic mouse, among those under active evaluation by the CTP (NIEHS, 1997). This review concludes with additional supportive evidence and references to explain the appearance of lymphomas in the original mouse bioassay (NTP, 1996) and in the subsequent mechanistic study with the *p53+/-* transgenic mouse assay (Tice and Furedi-Machacek, 1997).

2.0 CARCINOGENICITY

Two-year carcinogenicity studies were conducted on phenolphthalein on the basis that this chemical was a high-volume OTC pharmaceutical for which there were few rodent or human data available (NTP, 1996).

In the study, groups of 50 male and 50 female F344/N rats were administered phenolphthalein in the diet for 2 years at concentrations of 0, 12,000, 25,000, and 50,000 ppm (equivalent to average daily doses of approximately 500, 1,000, or 2,000 mg phenolphthalein/kg body weight in males and 500, 1,000, or 2,500 mg/kg in females). Similar numbers of $B6C3F_1$ mice of each sex also were administered phenolphthalein in the diet for 104 weeks, but at concentrations of 0, 3,000, 6,000, and 12,000 ppm (equivalent to average daily doses of approximately 300, 600, or 1,200 mg phenolphthalein/kg body weight in males and 400, 800, or 1,500 mg/kg in females). The doses were selected on the basis of previously conducted 14-day and 13-week studies (Dietz *et al.*, 1992). Dosing was initiated in the rats and mice at the age of 6 to 7 weeks old and continued in the daily diet for 104 weeks in these lifetime studies.

Summaries and discussions of the salient pathological findings reported in the carcinogenicity study and of the results of the genetic toxicology tests are presented in the following sections.

3.0 RESULTS OF THE NTP (1996) CARCINOGENICITY STUDY

3.1 F344/N Rats

3.1.1 Survival, Body Weights, and Clinical Findings

Survival of exposed males and females was similar to that of the controls. The mean body weights of exposed males were less than those of the controls through most of the second year of the study, and the mean body weights of exposed females were less than those of the controls from about week 16 until the end of the study. Clinical findings attributed to phenolphthalein exposure included thin appearance and ruffled fur in all exposed groups of males.

3.1.2 Pathology Findings

A summary of the pathological findings in F344 rats is presented in Table 1.

Table 1 Incidence of Selected Proliferative Lesions in Rats in the NTP Study

Observation	Males				Females			
	0 ppm	12,000 ppm	25,000 ppm	50,000 ppm	0 ppm	12,000 ppm	25,000 ppm	50,000 ppm
Adrenal gland								
benign pheochromocytoma	17/50	34/50	34/50	34/50	3/50	11/50	9/50	2/49
benign + malignant pheochrom.	18/50	35/50	35/50	35/50	3/50	12/50	10/50	2/49
focal medullary hyperplasia	13/50	22/50	18/50	23/50	10/50	18/50	15/50	11/50
Kidney								
tubular adenoma (standard + extended evaluations)	1/50	10/50	15/50	15/50	1/50	2/50	0/50	1/50
tubular adenoma or carcinoma (standard + extended evaluations)	1/50	10/50	16/50	16/50	1/50	2/50	0/50	1/50
tubular hyperplasia	3/50	25/50	29/50	27/50	1/50	4/50	3/50	3/50

Table 1 Incidence of Selected Proliferative Lesions in Rats in the NTP Study

Observation	Males				Females			
	0 ppm	12,000 ppm	25,000 ppm	50,000 ppm	0 ppm	12,000 ppm	25,000 ppm	50,000 ppm
nephropathy (average grade)	1.8	2.9	3.1	3.1	1.2	1.4	1.5	1.5

Note: shaded cells indicate significant difference from control value

As can be seen from Table 1, the incidences of benign pheochromocytoma of the adrenal medulla in all exposed groups of males were significantly greater than those in the controls. The incidences of benign pheochromocytoma in 12,000 ppm females and of benign or malignant pheochromocytoma (combined) in 12,000 and 25,000 ppm females were significantly greater than those in the controls. The numbers of exposed males with bilateral benign pheochromocytomas exceeded the number of controls with these neoplasms. The incidences of malignant pheochromocytomas in exposed rats were similar to those in the controls. The incidences of focal hyperplasia of the adrenal medulla in the 12,000 and 50,000 ppm males were significantly greater than in the controls. It is interesting to note that in females the incidence of benign pheochromocytoma, benign and malignant pheochromocytoma and of focal medullary hyperplasia was decreased in a dose-dependent fashion in the mid- and high-dose groups compared to the low dose group. The incidences of these lesions in the high-dose group were essentially the same as the controls.

The incidences of renal tubular cell adenoma in 50,000 ppm male rats and of renal tubular cell adenoma or carcinoma (combined) in 12,000 and 50,000 ppm male rats were significantly greater than those in the controls. Although the increased incidences were predominantly of renal tubular cell adenoma, four carcinomas were observed in exposed males (0 ppm, 0/50; 12,000 ppm, 1/50; 25,000 ppm, 1/50; 50,000 ppm, 2/50). The incidences of renal tubular cell neoplasms in exposed groups of females were similar to those in the controls. The findings from an extended evaluation (step section) of the kidneys of female rats were similar to those from the standard evaluation. The incidences of nephropathy in all exposed groups of females were significantly greater than in the controls, and the severity of nephropathy in all exposed groups of males and in 25,000 and 50,000 ppm females was significantly greater than in controls.

In addition to the findings reported in Table 1, the incidences of diffuse hyperplasia of the parathyroid gland (0/41, 16/48, 14/49, 14/46), fibrous osteodystrophy of the femur (0/50, 17/50, 14/50, 12/50), and mineralization (0/50, 11/50, 5/50, 5/49) and degeneration (0/50, 11/50, 5/50,4/49) of the glandular stomach in exposed groups of males were generally significantly greater than those in the controls. The incidences of hyperplasia of the thyroid gland C-cells (13/50, 3/50, 9/49, 4/49) in the 12,000 and 50,000 ppm males were significantly less than in controls. The above described changes are commonly observed in male rats with advancing nephropathy and are considered to be associated with a calcium/phosphorus imbalance created by compromised functional capacity of the kidney and/or possibly as a result of an increase in calcium absorption. These data, in particular the finding of fibrous osteodystrophy of the femur, mineralization of the stomach and aorta and evidence of reduced function of thyroid C-cells (Roe, 1993) suggest the development of a treatment-related hypercalcemic state in the male rats.

An irritant effect of phenolphthalein was apparent in the forestomach of males, with increased incidences of chronic inflammation and hyperplasia of the forestomach epithelium reported in the two highest dose groups.

3.2 B6C3F$_1$ Mice

3.2.1 Survival, Body Weights, and Clinical Findings

Survival of the 12,000 ppm females was significantly lower than that of the controls; survival of all other exposed groups of mice was similar to that of the controls. The mean body weights of 12,000 ppm males were slightly less than those of the controls beginning at week 93 of the study, and the mean body weights of the 3,000, 6,000, and 12,000 ppm females were less than those of the controls during most of the second year of the study. In exposed mice, there were no clinical findings related to phenolphthalein exposure.

3.2.2 Pathology Findings

The pathological findings in B6C3F$_1$ mice are presented in Table 2.

Table 2 Incidence of Selected Pathological Observations in Mice in the NTP Study

Observation	Males				Females			
	0 ppm	3,000 ppm	6,000 ppm	12,000 ppm	0 ppm	3,000 ppm	6,000 ppm	12,000 ppm
Haematopoietic and General Body System								
malignant lymphoma (all types)	6/50	8/50	12/50	8/49	15/50	28/50	33/50	25/50
malignant lymphoma (thymic origin)	0/50	4/50	7/50	2/49	1/50	9/50	10/50	7/50
histiocytic sarcoma	1/50	3/50	11/50	12/49	0/50	2/50	7/50	7/50
bone marrow myelofibrosis	3/50	8/50	8/50	19/49	34/50	34/50	38/50	36/50
bone marrow pigmentation	0/50	2/50	5/50	16/49	2/50	3/50	11/50	11/50
Thymus								
atypical hyperplasia	0/50	3/50	7/50	7/50	0/50	7/50	6/50	5/50
Ovary								
benign sex-chord stromal tumor	NA	NA	NA	NA	0/50	7/49	6/50	5/50
hyperplasia	NA	NA	NA	NA	4/50	11/49	10/50	17/50
Kidney								
hyaline droplet accumulation	0/50	2/50	5/50	5/49	0/50	1/50	3/50	8/50
Testis								
degeneration of germinal epithelium	1/50	49/50	50/50	47/48	NA	NA	NA	NA

Note: shaded cells indicate significant difference from control value

The incidences of histiocytic sarcoma, a malignancy of the macrophage/phagocyte system, in 6,000 and 12,000 ppm males and females were significantly greater than those in the controls and occurred with a significant positive trend. In this study, histiocytic sarcoma was consistently observed in the liver with several other sites (*e.g.*, spleen, lung, bone marrow, and various lymph nodes) involved less frequently. The incidence of hyaline droplet accumulation in the kidney, an effect associated with histiocytic sarcoma due to the release of lysozyme from the affected macrophages (Hard and Snowden, 1991), was increased in males treated at 6,000 and 12,000 ppm and in females treated at 12,000 ppm.

The incidences of all types of malignant lymphoma and of lymphoma of thymic origin in all exposed groups of females were significantly greater than those in the controls while the incidences of all types of malignant lymphoma in all exposed groups of males were similar to that in the controls. The incidences of lymphoma of thymic origin were increased in exposed groups of males, but were significantly increased only in the 6,000 ppm group. The incidences of atypical hyperplasia of the thymus in 6,000 and 12,000 ppm males and in all exposed groups of females were significantly greater than those in the controls.

In the exposed mice there was evidence of bone marrow/haematopoietic system toxicity. Increased incidences of myelofibrosis of the bone marrow were found in 12,000 ppm males (0 ppm, 3/50; 3,000 ppm, 8/50; 6,000 ppm, 8/50; 12,000 ppm, 19/49) and an increased severity, but not incidence, of this lesion was reported in exposed females. There also were increased incidences of pigmentation of minimal to mild severity in the bone marrow of 6,000 and 12,000 ppm males (0/50, 2/50, 5/50, 16/49) and females (2/50, 3/50, 11/50, 11/50). In addition, the incidences of haematopoietic cell proliferation in the red pulp of the spleen (10/50, 22/50, 28/50, 21/49) in all exposed groups of males were significantly greater than in the controls, and the severity of this lesion increased with increasing exposure concentration. These data indicate a toxic effect of high-dose phenolphthalein exposure on the haematopoietic system.

The incidences of benign sex-cord stromal tumors of the ovary in exposed females and of hyperplasia of the ovarian epithelium in the 3,000 and 12,000 ppm groups were significantly greater than in the controls. In dosed males, the incidence of germinal epithelial degeneration of the testis was significantly greater than in controls.

It is interesting to note that the incidences of hepatocellular adenoma in all exposed groups of males and females, and of hepatocellular adenoma or carcinoma (combined) in 6,000 and 12,000 ppm males and all exposed groups of females, were significantly less than those in the controls, and these lesions occurred with significant negative trends. Multiple hepatocellular adenomas were observed more frequently in the control groups than in the exposed groups. The incidence of clear cell and eosinophilic foci in all exposed groups of males and of mixed cell foci in 12,000 ppm males were significantly less than those in the controls. The incidences of eosinophilic foci in exposed groups of females were significantly less than that in the

controls. In addition to the liver, the incidences of proliferative lesions of the pituitary and thyroid glands in female mice were reported to be reduced by phenolphthalein treatment.

4.0 DISCUSSION OF THE CARCINOGENICITY RESULTS

4.1 Plasma Concentrations of Phenolphthalein in Rodents and Adults

Prior to any discussion of what the results of the NTP (1996) carcinogenicity study mean to humans, it is important to consider the differences between the doses received by the rats and mice in the NTP study and the doses likely achieved in humans through therapeutic use. Generally, for pharmaceutical products, dosing in a 2-year lifetime study which achieves blood or plasma area-under-the-curve (AUC) values 25-fold greater than the AUC expected in humans at therapeutic doses is considered sufficient to represent the high dose in the evaluation of the carcinogenic potential of non-genotoxic compounds with similar metabolic profiles in humans and the test species (FDA, 1994).

A summary of the plasma concentrations of phenolphthalein, and resultant AUC values, achieved in rats and mice in the NTP study is shown in Tables 3 and 4.

Table 3 Plasma Concentrations of Phenolphthalein in Rats in the NTP Study

Pharmacokinetic Parameters	Males			Females		
	12,000 ppm	25,000 ppm	50,000 ppm	12,000 ppm	25,000 ppm	50,000 ppm
Plasma concentration (average @ 6 am) (μg/ml)	107.8	127	177.2	94.1	90.1	104.2
Plasma concentration (average @ 9 pm) (μg/ml)	69.2	59.5	80.9	69.2	110.8	83.3
AUC (6 am to 9 pm) (μg•hr/ml)	1131	1087	2173	1268	1444	1422

Table 4 Plasma Concentrations of Phenolphthalein in Mice in the NTP Study

Pharmacokinetic Parameters	Males			Females		
	3,000 ppm	6,000 ppm	12,000 ppm	3,000 ppm	6,000 ppm	12,000 ppm
Plasma concentration (average @ 6 am) (μg/ml)	128.7	149	174	112	180.6	233.1
Plasma concentration (average @ 9 pm) (μg/ml)	102	121.3	175.7	134.5	112.3	203.3
AUC (6 am to 9 pm) (μg•hr/ml)	1818	1776	2065	1815	2066	2804

For both rats and mice, NTP (1996) concluded that the mean plasma concentrations of total phenolphthalein (free and conjugated) after 2 years of exposure varied little with time of day. NTP (1996) also concluded that the plasma concentrations of total phenolphthalein were approximately the same between exposure groups and between sexes. Also, although the mice were exposed to much lower dietary concentrations of phenolphthalein, but similar doses on a body weight basis, the achieved plasma concentrations were similar to or slightly greater than those achieved in rats. These data indicate that absorption mechanisms were saturated, or nearly saturated, in the rat and mouse at the lowest dose levels tested of 12,000 and 3,000 ppm in the diet, respectively.

In the first Carcinogen Assessment Committee (CAC) meeting convened to discuss the results of the NTP carcinogenicity study, Dr. Choudary of the FDA noted that the achieved dose levels (500, 1000, 2000 mg/kg) were excessive based on the plasma concentrations achieved and the reported toxicities.

In a study of the pharmacokinetics of phenolphthalein in 4 healthy human volunteers, Selim and Luke (1992) reported that following a single 150 mg oral dose (approximately 2 mg/kg body weight), the plasma concentrations of phenolphthalein for the following 24 hours were generally in the range of 1 to 2 μg/ml, with peak values of up to 3 or 4 μg/ml, and minimal values in the range of 0.3 to 0.5 μg/ml. Based on these data, the plasma phenolphthalein concentrations and the resulting AUC values for the rat were 50- to 100- fold higher than

those likely to be achieved in humans at therapeutic doses. Clearly, based on the plasma concentration data, the dosing in the NTP study was excessive relative to normal human exposure and thus, any extrapolation of these results in rodents possible effects in human should be conducted with caution.

The large differences in duration of exposure and in the exposure received by the animals (starting at ages 6 to 7 week) in the lifetime NTP study and the potential exposure of adults as a result of the use of phenolphthalein as a laxative, must be kept in mind in the following discussions of the potential relevance to humans of the various tumor types reported to be increased by phenolphthalein treatment in the lifetime NTP study. Toxicities associated with extreme exposure levels in the NTP study are not evident at lower doses. Increased incidences of certain tumor types linked to such lifetime high-dose toxicity, or to another mechanism(s) operative only at high-doses, have no relevance to adults exposed at therapeutic doses.

Plasma concentrations of phenolphthalein were also measured in female *p53+/-* transgenic mice exposed to phenolphthalein in the diet for 24 weeks (see Appendix A). While these data indicate that the transgenic animals absorbed the drug and confirm systemic exposure, the use of these data from sensitized research animals to compare to human exposure in a quantitative manner is not appropriate. The *p53+/-* transgenic mouse is not intended for use in quantitative risk assessment, but is an alternative model under evaluation for providing insight into the mechanisms of tumor development.

4.2 Pheochromocytomas in Rats

In evaluating the significance to humans of the pheochromocytomas reported to occur in F344 rats exposed to high concentrations of phenolphthalein, one must be cognizant of the important differences between rats and humans in spontaneous proliferative lesions which normally develop in the adrenal medulla. These differences, which provide strong evidence to indicate that the increased incidence of pheochromocytomas seen following high-dose exposure to phenolphthalein is specific to the rat, are briefly summarized below.

The most notable difference between rats and humans is the frequency with which spontaneous lesions of the adrenal medulla develop. In the rat, the spontaneous incidences of proliferative

lesions of the adrenal medulla, including diffuse and focal hyperplasia, as well as pheochromocytomas, are considerably greater and show much more variability than similar lesions in the normal human population (Cheng, 1980; Thompson *et al.*, 1981; Strandberg, 1983; Manger *et al.*, 1985; Roe and Bär, 1985; Tischler *et al.*, 1985, 1989; Tischler and DeLellis, 1988; Duprat *et al.*, 1990; Hamlin and Banas, 1990; Capen *et.al.*, 1991). The spontaneous incidence of tumors of the adrenal medulla in rats ranges from 0.5% in the Holtzman rat strain (Schardein *et al.*, 1968) to 17% in the F344 rat (Sher *et al.*, 1982) and to 69% in the Wistar rat (Pollard and Luckert, 1989). In contrast, the spontaneous incidence in humans has been reported to range from 0.005 to 0.1% (Minno *et al.*, 1954; von Schlegel, 1960; Symington, 1969; Manger *et al.*, 1985), with most data favoring the lower part of this range. The difference between rats and humans in the occurrence of spontaneous lesions of the adrenal medulla may relate to the unique sensitivity of rat chromaffin cells to mitogenic stimuli or to several morphological and functional differences between rat and human chromaffin cells.

Secondly, pheochromocytomas are inducible in rats by many pharmacologically unrelated substances (Tischler *et al.*, 1989), but have never been reported to be associated with any substance in humans. Induction of pheochromocytomas in rats appears to reflect an exacerbation of their tendency for spontaneous development (Tischler *et al.*, 1989).

Thirdly, in the rat, catecholamine-secreting adrenal medullary chromaffin cells have been demonstrated to be capable of proliferation following post-natal differentiation (Coupland and Tomlinson, 1989; Tischler *et al.*, 1989; Tischler and Coupland, 1994); however, in humans, mitoses in mature chromaffin cells are very rare (Coupland, 1969; Tischler *et al.*, 1989). Also, the proliferation of chromaffin cells in the adult rat has been shown *in vitro* to be induced by mitogenic agents which activate protein kinases or adenylate cyclases, while the chromaffin cells of humans are resistant to these mitogenic stimuli (*i.e.*, rat adrenal medullary cells remain responsive to mitogenesis throughout the life of the animal) (Tischler, 1992; Tischler and Riseberg, 1993). These data indicate that cells of the rat adrenal medulla are much more susceptible to mitogenic influences than are corresponding cells in humans.

Finally, the rat adrenal medulla is sensitive to the effects of increased calcium absorption (hypercalcemia). Many substances appear to increase the incidence of pheochromocytoma in

the rat *via* a mechanism involving alteration of calcium homeostasis (Lynch *et al.*, 1996). Recent work by Tischler *et al.* (1996) has strengthened the hypothesis that altered calcium homeostasis is involved in the development of the rat adrenal medullary proliferative lesions. Tischler *et al.* (1996) demonstrated that administration of either 20,000 or 40,000 I.U. of vitamin D_3 to groups of six male Sprague-Dawley rats resulted in dramatic increases in the serum calcium concentrations and concomitant increases in the frequency of labeled chromaffin cells as measured by bromodeoxyuridine incorporation.

In the NTP study, the adrenal pheochromocytomas clearly were associated with a number of clinical and histopathological observations consistent with the development of a hypercalcemic state, including: diffuse hyperplasia of the parathyroid gland, fibrous osteodystrophy of the femur, and mineralization of the glandular stomach in exposed males. There also was a decreased incidence of thyroid C-cell (calcitonin-producing cells) hyperplasia in mid- and high-dose males, likely due to the reduced activity of these cells in response to elevated serum calcium levels. The hypercalcemic state can be explained by increased calcium absorption and/or decreased excretion resulting from the treatment-related exacerbation of chronic progressive nephropathy.

In summary, the striking differences between rats and humans in the incidence of spontaneous and induced adrenal medullary lesions, the morphological and functional differences between the lesions themselves, and the likely involvement of a rat-specific mechanism of action (*i.e.*, hypercalcemia), strongly indicate that the adrenal medullary proliferative lesions reported in the NTP study are of no relevance to humans. These conclusions regarding their relevance to humans of the pheochromocytomas are essentially in agreement with the comments on the NTP study provided by Dr. Frances Roe in an earlier report (Roe, 1996).

4.3 Renal Tubular Adenomas in Male Rats

The renal tumors (mostly benign) reported to occur in male, but not female rats, were associated with an increased incidence of hyperplasia and increased severity of chronic progressive nephropathy, a common age-related lesion in the F344 rat (Montgomery and Seely, 1990). The progression to neoplasia from chronic progressive nephropathy in the rat

has been recognized as a species-specific phenomenon caused by a non-genotoxic mechanism (Hard, 1990; Montgomery and Seely, 1990; Alden and Frith, 1991).

The pathology data point to a role of high-dose toxicity in the development of these neoplasms, especially given that the male F344 rat is known to be much more sensitive than the female to the development of both nephropathy and renal tubular neoplasms (Montgomery and Seely, 1990). Also, the report of an increased incidence of renal tubular cell neoplasms is consistent with exacerbation of chronic nephropathy brought about by disruption of calcium homeostasis and by toxic effects of high-dose chemical exposure (Montgomery and Seely, 1990; Roe, 1993). The marked increase in the incidence of renal tubular cell hyperplasia in treated males further supports a mechanism involving toxicity-related induction of cell proliferation. The role of a genotoxic metabolite is considered unlikely given that no increase in renal tubular cell neoplasms occurred in female rats and given that there is no evidence indicating the existence of significant sex-dependent differences in the metabolism of phenolphthalein which could account for the development of the renal neoplasms in male animals only.

In the first meeting of the CAC, it was generally agreed that exacerbation of chronic progressive nephropathy could result in the reported increased incidence of benign renal tubular cell tumors in male rats, and generally agreed that these tumors were likely not predictive of a carcinogenic risk to humans. At this meeting, Dr. Choudary of the FDA also indicated that the adrenal medullary and renal tubular cell tumors were likely the consequence of toxicity inflicted with excessive doses and do not necessarily indicate carcinogenic risks to humans.

Overall, the pathological data support the conclusion that the renal tubular cell tumors in the male rat, most of which were benign, were the result of high-dose toxicity and exacerbation of chronic progressive nephropathy, a condition in the advanced stages which is known to lead to an increased incidence of these neoplasms by a non-genotoxic mechanism (Hard, 1990; Montgomery and Seely, 1990; Alden and Frith, 1991). In an earlier review of the NTP study, Roe (1996) also concluded that the renal tubular cell neoplasms could readily be explained by the apparent effects of phenolphthalein on calcium homeostasis and the resultant acceleration of chronic progressive nephropathy (Roe, 1996).

4.4 Ovarian Tumors in Mice

In the NTP 2-year lifetime study, the incidence of ovarian sex chord stromal tumors was increased, although not in a dose-dependent fashion. All of these tumors were benign. A similar increase in the incidence of sex chord stromal cell hyperplasia accompanied the tumor increases in all treated groups. These observations are not consistent with a genotoxic mode of action. Instead, the data are more consistent with the presence of a promoting influence whereby there is the development of a continuum of proliferative lesions ranging from hyperplasia, through to benign tumors and, in a few cases, to a low incidence of malignant tumors. It is possible that the estrogenic activity of phenolphthalein resulted in the promotion of these tumors, although it is recognized that not all estrogenic chemicals produce increases in the incidence of the type of ovarian tumors reported in the NTP study.

The results of the *p53+/-* transgenic mouse study (Tice and Furedi-Machacek, 1997) further support a mechanism not involving genotoxicity. No ovarian tumors or any other evidence of a proliferative effect on this organ, were reported in this study (Tice and Furedi-Machacek, 1997).

The likelihood of a hormonal mechanism of action is strengthened by the observations of a variety of changes in the proliferative lesions of various endocrine tissues of the treated mice in the NTP study. The incidences of hyperplasia of the pituitary gland (11/49, 4/50, 1/49, and 1/48, in the control, 3,000, 6,000, and 12,000 ppm groups, respectively) and of follicular cells of the thyroid gland (27/50, 8/50, 3/50, and 7/50, in the control, 3,000, 6,000, and 12,000 ppm groups, respectively) were markedly reduced in treated females. Also, the incidences of hepatocellular adenoma (22/50, 12/50, 8/50, and 10/49 in the control through high-dose males, respectively, and 17/50, 2/50, 6/50, and 1/50 in the control through high-dose females, respectively) and of hepatocellular adenoma and carcinoma combined were decreased in exposed mice of both sexes. The incidences of foci of cellular alteration were similarly decreased in both sexes. The incidences of pre-neoplastic and neoplastic lesions of the liver, which in the $B6C3F_1$ strain of mice are highly susceptible to certain non-genotoxic and also genotoxic carcinogens, would have been increased, rather than decreased, if phenolphthalein indeed possessed genotoxic activity. The reduced incidence of these tumors likely reflects the apparent change in the hormonal milieu associated with phenolphthalein treatment.

The results of the NTP study show a clear effect of phenolphthalein on the hormonal status of female mice and provide histopathological evidence of a promotional mechanism (*e.g.*, observation of hyperplasia and benign tumors only). Given these data, the demonstrated estrogenic activity of phenolphthalein, and the absence of proliferative lesions of the ovaries in the transgenic mouse assay (Tice and Furedi-Machacek, 1997), the ovarian tumors are concluded not to have developed by a mechanism involving genotoxicity.

4.5 Histiocytic Sarcomas in Mice

At the second meeting of the CAC in April, 1997, the increased incidence of histiocytic sarcomas a neoplasm of the macrophage/monocyte cell series (Frith *et al.*, 1980; Pattengale and Frith, 1986; Ward and Sheldon, 1993) appeared to have been considered likely the result of genotoxic mechanisms. A review of the histopathological data from the NTP study, however, does not directly support this deduction. First, these neoplasms were associated with marked increases in hematopoietic system toxicity, particularly toward myeloid components (NTP, 1996). In the high-dose males, there was a large increase in bone marrow myelofibrosis, indicative of cytotoxic effects on bone marrow progenitor cells. Bone marrow pigmentation also was increased in the mid- and high-dose animals of both sexes. Bone marrow toxicity also was evident in the preliminary 13-week toxicity study (Dietz *et al.*, 1992). High-dose toxicity particularly toward bone marrow precursor cells, and the resultant rapid cell replication, as seen in the red pulp of the spleen, conceivably could render hematopoietic cells more susceptible to malignant transformation.

Potentially related to the hematopoietic system toxicity and the development of histiocytic sarcomas in mice is the demonstrated weak estrogenic activity, both *in vitro* and *in vivo* (Bitman and Cecil, 1970; Ravdin *et al.*, 1987; Nieto *et al.*, 1990), of phenolphthalein. Estrogens are known to have immunomodulating properties (Luster *et al.*, 1984, 1985) and have been demonstrated to be a cause of bone marrow myelofibrosis, bone marrow hypoplasia and splenic hematopoiesis (Fried *et al.*, 1974; Adler and Trobaugh, 1978; Sass and Montali, 1980; Highman *et al.*, 1981), effects all observed in response to high-dose exposure of mice to phenolphthalein. Histiocytic sarcomas in mice have been documented to occur in response to treatment with other non-genotoxic pharmaceutical products, including goserelin and histrelin (Davies and Monro, 1995; PDR, 1996). These products, gonadotropin releasing hormone

agonists, also produce tumors in other hormonally responsive sites (PDR, 1996). The estrogenic activity of phenolphthalein may in fact account for the "high-dose" toxicity to the hematopoietic system in the mouse. The estrogenic activity of phenolphthalein in the NTP study was evidenced by the degeneration of the germinal epithelium of the testes in exposed male mice.

It is interesting to observe, in the *p53+/-* transgenic mouse assay conducted by Tice and Furedi-Machacek (1997) to investigate the potential role of a genotoxic mechanism in the development of the hematopoietic system and ovarian tumors, that no evidence of histiocytic sarcoma development was found in any of the treated mice, even those treated at 3,000 and 12,000 ppm in the diet, the same concentrations as used in the NTP study. The lack of induction of these tumors in this system indicates that either the *p53+/-* mouse model is insensitive to the development of histiocytic sarcoma or, more likely, that these tumors in the NTP (1996) study arose through non-genotoxic mechanisms (*e.g.*, high-dose toxicity, estrogenic effects) which require longer treatment times than provided by the dosing regimens used in this assay.

In summary, the histological data showing evidence of high-dose toxicity and weak estrogenic activity indicate that the histiocytic sarcomas in mice developed as the result of non-genotoxic mechanisms of action. The lack of induction of histiocytic sarcomas in the *p53+/-* assay Tice and Furedi-Machacek (1997) further supports a non-genotoxic mode of action.

4.6 Malignant Lymphomas in Mice

One of the more significant findings in the 2-year mouse study was the increased incidence of malignant lymphoma, particularly of thymus-derived lymphoma in treated females and in the mid-dose males.

Following the second meeting of the CAC, there appeared to be a general consensus that the thymic-derived lymphomas in treated mice were likely the result of genotoxicity. A critical evaluation of the pathological findings and other recent experimental evidence, however, does not support this position.

It is possible that the weak estrogenic activity of phenolphthalein may account for the development of both the histiocytic sarcomas and the thymic-derived lymphomas. This hypothesis was raised previously in Dr. Roe's (Roe, 1996) review of the NTP study results. Estrogenic action has an immunomodulatory influence, generally depressive on the thymus and stimulatory to cells of the reticuloendothelial system (Forsberg, 1984; Luster *et al.*, 1984). Steroidal estrogens have been reported to cause the development, often rapid, of lymphoid tumors in several strains of mice (reviewed in IARC, 1979). The exact mechanism(s) by which estrogenic activity is involved in the development of lymphoid tumors is unclear, but is unlikely to involve genotoxicity. As previously indicated, in the NTP study there was clear evidence of estrogenic activity of phenolphthalein in the form of degeneration of the germinal epithelium of the testes in almost all exposed males.

As with the histiocytic sarcomas, in the NTP study, doses which produced an increased incidence of thymic-derived lymphomas were associated with clear signs of hematopoietic system toxicity, particularly in the males (bone marrow myelofibrosis, pigmentation, hematopoiesis of the red pulp of the spleen, and bone marrow hypoplasia). These observations tend to support an estrogenic mechanism; however, the hematopoietic system toxicity observed appeared mostly associated with myeloid- and erythroid-derived cells (NTP, 1996), cell types more closely related to the development of histiocytic sarcoma, rather than lymphoid precursor cells, the cell type involved in the development of thymic lymphoma.

In the Tice and Furedi-Machacek (1997) *p53*+/- transgenic mouse assay, thymic-derived lymphomas were markedly increased in female mice treated at 3,000 and 12,000 ppm in the absence of any clear signs of hematopoietic system toxicity. These results would seem to argue against a mechanism directly related to high-dose tissue toxicity. An analysis of the *p53* gene in cells from the thymic lymphomas, however, revealed that this gene was lost in 21 of the 21 cases analyzed rather than mutated (Dunnick *et al.*, 1997). A mutated *p53* gene would have been expected for a "classical" genotoxic carcinogen (CanTox, 1997; attached as Appendix A). This observation, along with the results of the other genotoxicity tests, for example, the finding of a preponderance of kinetochore material in the micronuclei of phenolphthalein-treated *p53*-deficient mice (Tice and Furedi-Machacek, 1997), show that an aneugenic mechanism is involved in the development of micronuclei and of the thymic lymphomas (CanTox, 1997; attached as Appendix A). The finding of an increased incidence

of atypical hyperplasia (cells showing replicative and morphological changes), a lesion considered by NTP (1996) to be pre-neoplastic or actual early lymphoma, in the thymus of treated mice also is consistent with an aneugenic mechanism. Aneugenesis could be expected to result in the development of cells in various stages of transformation to a malignant state.

Evidence for such a threshold is seen in the results of the transgenic mouse assay (Tice and Furedi-Machacek, 1997). At doses of 3,000, and 12,000 ppm, doses which saturate absorption mechanisms in B6C3F$_1$ mice, the reported incidence of thymic lymphoma was 17/20 and 14/20, respectively. At these doses, the plasma concentrations achieved (126 and 271 μg/ml, respectively) were similar to those achieved in mice in the 2 year NTP study. At lower doses of 200, 375 and 750 ppm (equivalent to 43, 84, and 174 mg/kg body weight/day and associated with plasma concentrations of 38, 64, 89 μg/ml, respectively), the incidences of these neoplasms were much lower at 1/20, 0/20, and 2/20, respectively. These incidence values were not significantly different from the controls.

Overall, the histopathological data from the NTP (1996) study, the results of various genotoxicity tests, as well as of the *p53*+/- transgenic mouse assay (Tice and Furedi-Machacek, 1997), and the analysis of the loss of the *p53* gene in the thymic lymphomas in the transgenic mouse study (Dunnick *et al.*, 1997), indicate that an aneugenic rather than a genotoxic mechanism is involved in the development of the thymic lymphomas reported to occur in mice in the NTP (1996) study. Based on the results of carcinogenicity and aneugenicity studies with estrogens, the estrogenic activity of phenolphthalein likely played a role in the development of the thymic lymphomas through an aneugenic mechanism. The evidence for this conclusion is discussed more fully in a later section in this report. In either case, the mechanism would be expected to be threshold-dependent (*e.g.*, receptor mediated or expressed only at high-doses). An aneugenic mechanism is expected to have a threshold of exposure below which no adverse effects would occur (Parry *et al.*, 1994). For human therapeutic doses, which are likely to be well below the threshold dose, there appears to be negligible risk for the development of these neoplasms.

5.0 CONCLUSIONS ABOUT PHENOLPHTHALEIN CARCINOGENICITY

On the basis of the minutes of the first and second CAC meetings, convened to discuss the NTP report on phenolphthalein and other relevant data, the findings of greatest concern appeared to be the reported increased incidences of ovarian tumors and of histiocytic sarcomas and thymic-derived lymphomas in mice. The neoplasms reported to occur in rats (pheochromocytomas and renal tubular adenomas) were generally considered not to present a carcinogenic risk to humans.

On the basis of the present review, the rat adrenal medullary pheochromocytomas are concluded to be the result of a rat-specific mechanism of action likely involving altered calcium homeostasis. Also, these tumors are considered of no relevance to humans due to the known differences between rats and humans in the incidence of spontaneous and induced adrenal medullary lesions and in the morphological and functional properties of the lesions themselves. The renal tumors in phenolphthalein-treated male rats are concluded to be the result of high-dose toxicity, including the exacerbation of chronic progressive nephropathy, a condition in the advanced stages which is known to lead to an increased incidence of renal tubular cell neoplasms (Hard, 1990; Montgomery and Seely, 1990; Alden and Frith, 1991).

With respect to the increased incidence of tumors reported in phenolphthalein-treated mice, there is evidence to indicate that they also were not the result of genotoxic mechanisms. First, with respect to the ovarian tumors, the histopathology data indicate a clear effect of phenolphthalein on the hormonal status of the female mice as well as evidence of tumor development *via* a hormonal mechanism (*i.e.*, observation of hyperplasia and benign tumors only). The demonstrated estrogenic activity of phenolphthalein and the absence of ovarian tumors in the *p53*-deficient mouse study (Tice and Furedi-Machacek, 1997) further demonstrate that these tumors did not arise by a genotoxic mechanism, but instead required long-term exposure to a weakly estrogenic substance.

Second, for the histiocytic sarcomas, histological data from the NTP study showed evidence of high-dose toxicity (*i.e.*, myelofibrosis, pigmentation, bone marrow hypoplasia, hematopoietic cell proliferation in the red pulp of the spleen) and of estrogenic activity. Estrogens are known to produce toxic effects in the hematopoietic system and to induce tumors of the

hematopoietic system in this species (reviewed in IARC, 1979). These observations indicate that the histiocytic sarcomas in mice in the NTP study developed as the result of a non-genotoxic mechanism of action. The lack of induction of histiocytic sarcomas in the *p53+/-* assay (Tice and Furedi-Machacek, 1997) further supports this conclusion.

Finally, the histopathological data from the NTP study, and the results of various genotoxicity tests and of the *p53+/-* transgenic mouse assay (Tice and Furedi-Machacek, 1997), indicate that an aneugenic mechanism is involved in the development of the thymic lymphomas in the NTP study, as discussed more thoroughly later in this report. Also, as with the histiocytic sarcomas, based on the results of carcinogenicity studies with estrogens, it is also possible that the estrogenic activity of phenolphthalein played a role in the development of the thymic lymphomas through the aneugenic mechanism. Evidence indicating altered hormonal status was seen in the observations of decreased incidences of proliferative lesions of the pituitary gland, thyroid gland, and liver. In any case, the mechanism involved in the development of the thymic lymphomas is expected to be threshold-dependent (*i.e.*, receptor mediated or expressed only at high-doses), with negligible carcinogenic risk to humans at therapeutic doses.

In conclusion, non-genotoxic and likely rodent-specific mechanisms can account for the tumors observed in the bioassays conducted by the NTP (1996) and in the alternative model by Tice and Furedi-Machacek (1997). The pathologic evidence which points to a non-genotoxic mode of action and the pharmacokinetic data demonstrating that the animals in the NTP (1996) study experienced plasma concentrations of phenolphthalein which were up to 100-fold greater than potentially attainable in humans exposed to therapeutic doses, provide convincing evidence to indicate that phenolphthalein presents no carcinogenic risk to humans at therapeutic doses.

6.0 NON-GENOTOXIC OR GENOTOXIC MECHANISM FOR MOUSE LYMPHOMAS

6.1 Results of Genotoxicity Studies Conducted Prior to the NTP (1996) Study

Prior to the conduct of the NTP (1996) study, the results of several NTP *in vitro* and *in vivo* genotoxicity tests had been reported in the literature (Mortelmans *et al.*, 1986; Dietz *et al.*, 1992; Witt *et al.*, 1995) in addition to other published reports. In bacterial systems, including assays using *Salmonella typhimurium* (Bonin *et al.*, 1981; Mortelmans *et al.*, 1986) and DNA damage repair-deficient strains of *Bacillus subtilis* (Kada *et al.*, 1972; Fujita *et al.*, 1976), phenolphthalein has been reported to be non-mutagenic.

In an *in vitro* cytogenics assay, phenolphthalein, at concentrations of up to 50 μg/ml in the absence of an exogenous source of metabolic activation did not induce an increase in chromosome aberrations in Chinese hamster ovary (CHO) cells. In the presence of the S9 liver fraction, at concentrations of 40 μg/ml or more, and following chemical-induced cell cycle delay, phenolphthalein induced a dose-dependent increase in the number of chromosome aberrations (Witt *et al.*, 1995). Most of the aberrations were chromosome breaks located at the distal end of chromosome X_q. The mechanism of this preferential breakage is unexplained, but has been reported to occur with other chemicals (Galloway *et al.*, 1985). Galloway *et al.* (1987) reported that the observation of preferential breakage in chromosome X_q occurs only in the presence of S9 and suggested that the effect may be mediated through high-dose toxicity or through some effect on chromatin packing. Both in the presence and in the absence of S9, phenolphthalein was found to have no effect in CHO cells on the incidence of sister chromatid exchange (SCE) at all doses tested (up to 50 μg/ml) (Witt *et al.*, 1995). Given the expectation that SCE would be found if there was induction of chromosome aberrations (NTP, 1996), the unexplained patter of X_q chromosome breakage does not provide convincing evidence of clastogenic activity in CHO cells.

In vivo, phenolphthalein has been shown to induce micronuclei (Dietz *et al.*, 1992; Witt *et al.*, 1995). Increased numbers of micronucleated polychromatic erythrocytes and/or miconucleated normochromatic erythrocytes were reported following analysis of blood samples collected from mice treated for 13 weeks with phenolphthalein in the diet at concentrations of 6,000 to

50,000 ppm (Dietz *et al.*, 1992). Similarly, Witt *et al.* (1995) reported that phenolphthalein administered to B6C3F1 mice at doses of 2,000 mg/kg body weight/day or greater for at least 2 days, induced micronuclei in erythrocytes. In Swiss CD-1 mice, lower doses of phenolphthalein of 120 mg/kg body weight/day were reported to be highly effective in inducing micronucleated erythrocytes when administered for 14 days.

6.2 Results of Genotoxicity Studies Conducted Following the NTP (1996) Study

Tsutsui *et al.* (1997) reported a series of results in a Syrian hamster embryo (SHE) cell system that showed evidence for cell transformation, chromosomal aberrations and mutation over a range of doses of phenolphthalein (10 to 40 μM). Previously, Kerckaert *et al.* (1996) had found that phenolphthalein was positive for cell transformation in SHE in a dose range of 60 to 80 μM phenolphthalein.

In a somewhat unusual step, Tsutsui *et al.* (1997) examined SHE cells for mutation at HPRT and Na^+/K^+ ATPase by selection for 6-thioguanine or ouabain resistance respectively (Table 1 of the manuscript submitted to the CAC). SHE cells are genetically stable and capable of metabolizing many chemicals to their ultimate mutagenic form (Kerckaert *et al.*, 1996). It is important to recognize that SHE cells are a mixed population, and therefore present a non-uniform target for mutation assays. Phenolphthalein was not mutagenic at Na^+/K^+ ATPase, but was reported to give a positive response at HPRT. We can not agree with this assessment, since all doses produced mutation frequencies below the control with the exception of the highest dose. The 40 μM dose was also the only dose that produced clear evidence of phenolphthalein toxicity in SHE cells (Figure 2 in the submitted manuscript). Tsutsui *et al.* (1997) gave a control mutation frequency for thioguanine resistance (Tg^r) of 4×10^{-6}, and an induced mutation frequency at the highest exposure (40 μM) of 5.6×10^{-6}. This does not even represent a doubling of the spontaneous value. It is quite possible that the increased number of thioguanine resistant cells in the nonuniform SHE cell population represents selective toxicity of a subpopulation of cells to phenolphthalein. In summary, Tsutsui *et al.* (1997) did not show data that support their conclusion that such a small increase in the number of TG^r should be considered significant.

Tsutsui *et al.* (1997) also examined 200 metaphases of SHE cells for chromosomal aberrations and found evidence of breaks, gaps and exchanges. They reported an increase in chromosomal aberrations at the dose (40 μM) that showed clear evidence of toxicity in the culture conditions. Similar results were reported for aberrations in CHO cells with microsomal activation (NTP 465; Witt *et al.*, 1995), but since there was no evidence of sister chromatid exchange in CHO cells, it was concluded that this result was equivocal.

A more interesting finding of Tsutsui *et al.* (1997) was the distribution of the chromosomal complement of SHE cells treated with 40 μM phenolphthalein for 72 hours. Table III of the submitted manuscript shows evidence of chromosomal loss as well as gain among the cells examined. Fifteen of the 100 metaphases examined showed evidence of aneuploidy or polyploidy after exposure to the highest dose of phenolphthalein. Such a result is consistent with the production of micronuclei found *in vivo*, and supports the hypothesis that phenolphthalein acts as a spindle poison, disturbing microtubule formation or attachment at metaphase and cytokinesis.

Finally, Tsutsui *et al.* (1997) found no evidence of DNA adducts in cells treated with phenolphthalein using a ^{32}P-postalbeling assay. This is not surprising, since Griffin *et al.* (1997) found some protein adducts, but no evidence that ^{14}C-phenolphthalein remained associated with tissues after *in vivo* exposures in rats and mice. Nevertheless, the absence of evidence for DNA adducts is relevant. Elsewhere Tice *et al.* (1997), using the SCGE assay, found no evidence for DNA breaks in leukocytes from mice treated with high doses of phenolphthalein *in vivo*. The production of stable DNA adducts would produce sites for DNA repair activity in leukocytes. Repair of DNA requires excision of the altered nucleotides, and this is readily detected by the SCGE assay (Fairbairn *et al.*, 1995). The fact that no adducts were detected lends additional support (Nestmann *et al.*, 1996) to the conclusion that phenolphthalein is not a direct DNA active compound.

To address the apparently conflicting nature of the genotoxicity data and to investigate the potential mechanisms involved in the development of the tumors reported to occur in mice in response to lifetime, high-dose treatment with phenolphthalein, additional studies (Tice and Furedi-Machacek, 1997; Dunnick *et al.*, 1997) were performed using a transgenic mouse model, the *p53+/-* deficient mouse.

As discussed more fully by Tennant *et al.* (1996), and summarized in CanTox (1997; attached as Appendix A), the *p53*-deficient mouse model is expected to respond to genotoxic, but not non-genotoxic, carcinogens. Therefore, having fully considered above the non-genotoxic mechanisms likely responsible for the rodent tumors found in the NTP (1996) study, including lymphoma in the mouse, it remains to discuss the malignant lymphomas found in the transgenic mouse.

A unique feature of the approach used in the program of the most recent NTP studies that attempt to characterize the potential carcinogenic effects from exposure to phenolphthalein is the commitment to analysis of mechanism of potential genotoxic action. These research findings (Tice and Furedi-Machacek, 1997) center around the responses observed in the *p53+/-* mouse and the analysis of these responses by sophisticated molecular biological techniques. These include the detection of micronuclei (MN) in circulating hemopoietic cells, the characterization of these micronuclei for kinetochore content using CREST antisera, and the analysis of peripheral blood cells for evidence of single or double strand breaks employing the exquisitely sensitive single cell gel electrophoresis (SCGE) assay. Additional data were produced from the analysis of lymphoma tissues from 21 tumors (Dunnick *et al.*, 1997) that showed clear evidence of loss, but not mutation, of the functional *P53* allele. This latter evidence indicates a non-genotoxic mechanism for tumor induction in thymic tissues of the mouse.

In this section, evidence is reviewed from recent, related laboratory investigations, leading to the conclusion that phenolphthalein acts as a weak aneugen and is not a direct genotoxin in the *p53+/-* mouse. This is an important distinction, since theoretically no accepted threshold exists for exposure to a genotoxic carcinogen, but there is clear scientific agreement that a threshold of exposure may be set for chemicals that interfere with mitosis by affecting microtubule assembly, chromosomal condensation or other functions at anaphase (Parry *et al.*, 1994).

First in this discussion, recent literature is reviewed which relates the induction of DNA damage to production of micronuclei by clastogenic action and to effects in the SCGE assay. Next, the CREST assay is described, which with other methods, including in situ fluorescence hybridization (FISH), has been applied to the analysis of micronuclei in mammalian cell

systems to differentiate the genotoxic actions of clastogens from the non-genotoxicity of aneugens. In this regard and since phenolphthalein has weakly estrogenic properties, it is also relevant to describe evidence linking the production of micronuclei by 17β-estradiol and diethylstilbestrol to their aneugenic activities as revealed by CREST and FISH analysis. Finally, results are described that show the complete loss of the *P53* allele observed in the thymic lymphoma tumors analyzed from *p53*+/- mice treated with high doses of phenolphthalein (Dunnick *et al.*, 1997). This analysis is more consistent with aneugensis leading to chromosome loss rather than genotoxicity resulting in mutation. The mechanism of action for DNA damage proposed by Sipe *et al.* (1997) from the biochemical analysis of the metabolism and disposition of phenolphthalein suggests production of hydroxyl radicals and active oxygen species. This mechanism is not consistent with the complete deletion of the *P53* gene.

6.2.1 Micronuclei and the SCGE Assay

As reported by Tice and Furedi-Machacek (1997), and discussed in CanTox (1997; Appendix A), micronuclei were induced in peripheral blood cells of phenolphthalein treated *P53* mice, but there was no evidence of DNA damage in the very sensitive SCGE assay. This apparent lack of correlation is discussed in light of findings with other chemicals.

An example of the correlation between the extent of DNA breakage measured in individual cells by the SCGE (comet) assay and micronuclei found at the same chemical exposures has been reported by Van Goethem *et al.* (1997). Human lymphocyte cultures were treated with cobalt (II), a metal divalent cation that is clastogenic, inducing chromosomal aberrations, micronuclei and sister chromatid exchanges in mammalian cell culture. The postulated mechanism responsible for these genotoxic effects is the production of hydroxyl radicals during metabolism in the presence of ionized cobalt. In addition to soluble cobalt, cobalt metal produced micronuclei in human lymphocytes in a dose dependent manner. Under the same conditions of exposure, alkaline SCGE assays revealed a similar dose dependent increase in single strand breaks as analyzed by both increased measured comet tail length (μm) and tail moment. The dose dependent increase in DNA breaks, including alkali labile sites (the result of depurination or depyrimidation events), is expected from cellular events that produce DNA damage by hydroxyl radical or active oxygen species.

The lack of evidence of single or double strand breaks in lymphocytes of the transgenic *p53+/-*mouse (Tice and Furedi-Machacek, 1997) examined by the SCGE assay shows that the micronuclei observed in this study arose by a mechanism of action other than clastogenic activity by phenolphthalein. If phenolphthalein exposure were to induce even a small amount of DNA damage or repair synthesis in lymphocytes, the SCGE assay would have sufficient sensitivity to detect it. The SCGE assay (see Appendix B attached) is extremely sensitive to the production of single strand breaks, so it is unlikely that significant levels of either oxygen or hydroxyl radicals were produced in the liver or other tissues as has been suggested (Griffin *et al.*, 1997; Sipe *et al.*, 1997). If these potent intermediates were produced with any abundance and transported to the interstitial fluids, circulating lymphocytes should have suffered some exposure, DNA breaks should have been produced in these cells and detected by the SCGE assay. There is no doubt that chemicals that produce micronuclei by clastogenic action are readily detected by the SCGE assay (Van Goethem *et al.*, 1997). This leads to the conclusion that the micronuclei induced in mice were not produced by a genotoxic mechanism that results in DNA damage detectable in the very sensitive SCGE assay.

6.2.2 Centromeric Labeling of Micronuclei

Dunnick *et al.* (1997) found that all (21/21) lymphoma tumors examined from *p53+/-* mice fed 3000 ppm phenolphthalein for six months had lost their heterozygosity for the tumor suppressor allele. If phenolphthalein were acting as a clastogen *in vivo*, one would anticipate that at least some of the tumors would arise from single site mutations within the *P53* gene rather than exclusively from complete loss of the wildtype allele from all tumor tissue.

Recently, experimental procedures have been developed to distinguish between micronuclei induced predominantly by clastogenic action from those resulting from chromosome loss or non-disjunction. These are the first methods that distinguish between aneugens, or chemicals that interfere with chromosome segregation events at cell division, and clastogens that simply cause DNA damage and break chromosomes. Until such methodologies were developed, there was virtually no nominal distinguishing feature(s) of micronuclei that contained an entire chromosome, or only part of one.

The sera of patients with the autoimmune disease Schleroderma CREST (Calcinosis, Raynaud's phenomenon, Esophageal dysmotility, Sclerodactyly and Telangiectasia) syndrome contain antibodies that specifically bind to the kinetochore region of chromosomes (Vig and Swerngin, 1986). This discovery has developed into the use of kinetochore specific antibodies to characterize the disposition of nuclear material in cells. Results with sera from these patients have shown that micronuclei induced by aneuploidy inducing agents principally contained CREST positive elements, while micronuclei induced by clastogens failed to react with antibodies under similar conditions of analysis (Schuler *et al.*, 1997). An analogous approach utilizes hybridization of fluorescent chromophore-tagged centromere-specific DNA sequences (FISH) to show the location of chromosomal elements in micronuclei (Elhajouji *et al.*, 1997; Schuler *et al.*, 1997).

Micronuclei arising from the aneugenic effect of spindle poisons as vincristine clearly have different content when compared to micronuclei resulting from the clastogenic effect of Mitomycin C (Sgura *et al.*, 1997). These authors showed that treatment of human lymphocytes *in vitro* with both chemicals produced dose dependent increases in micronuclei. Most micronuclei produced in response to vincristine treatment contained regions of DNA that hybridized to centromeric DNA probes. By contrast, only a minor fraction of the micronuclei induced by Mitomycin C contained centromere positive elements in micronuclei. Vincristine is a spindle poison that frequently leads to hyperploidy in daughter cells, and Sgura *et al.* (1997) concluded that the high frequencies of non-disjunction observed with human lymphocytes in culture could be due to failure of the centromere to divide at mitosis, or to non-functional attachment of spindle microtubules to one of the kinetochores. Marrazzini *et al.* (1994) have also shown that spindle poisons induce micronuclei, their work involving an *in vivo* mouse assay.

Another important, unifying body of evidence shows that compounds with estrogenic activity produced micronuclei through an aneugenic mechanism. *In vitro* treatment of Syrian hamster embryonic (SHE) cells with stilbene-type and steroidal estrogens induced dose related increases in the frequency of micronuclei. Diethylstilbestrol and 17β-estradiol (E2) induced micronuclei in SHE cells at a significantly elevated rate. A high percentage (92 to 99%) were CREST positive indicating a large number of micronuclei likely contained the greater part of whole chromosomes including the kinetochore. Similar cells treated with the clastogen 4-NQO

also produced micronuclei, but these rarely showed evidence of labeling with CREST antisera (Schiffman and De Boni, 1991; Schnitzler *et al.*, 1994). Micronuclei induced by DES or 17β-estradiol contained kinetochore positive elements, suggesting whole chromosomes, chromatids or centric fragments were in these extranuclear structures. It may be concluded that the likely primary cause of micronucleus induction by estrogenic substances in SHE cell cultures was a reflection of events caused by aneuploidy and not chromosome breakage.

Schnitzler *et al.* (1994) also noted that two different cell lines produced similar responses when treated with either DES or E2, and that micronuclei induced by either estrogenic compound contained chromosomal material to which kinetochores were attached. It was therefore concluded that it is the mode of action of the chemical estrogens, and not the cell type used, that is responsible for the elevated frequency of kinetochore positive micronuclei. Stopper *et al.* (1994) also found that DES and other aneugens including colcemid and vinblastine produced micronuclei in cultured mouse L5178Y cells, but selection for mutation at the thymidine kinase (TK) locus by the induction of resistance to triflurothymidine was unsuccessful. A high percentage (89 to 96%) of these micronuclei contained CREST positive kinetochore material (Stopper *et al.*, 1994). Evidence of Tucker and Barrett (1986) has shown that DES interacts with microtubules of the mitotic spindle apparatus in SHE cells, lending support to the hypothesis that DES increases the frequency of micronuclei by aneugenic activity and not as the result of direct DNA damage. Schuler *et al.* (1993) also found that 17β-estradiol induces a significant number of kinetochore positive micronuclei in SHE cells. Estradiol does not act as a clastogen, but the estradiol:receptor complex (E2:ER) does bind to the estrogen response element (ERE) which is located on the chromosomal DNA. It may alter the kinetochore structure or mask the epitope for the CREST antisera. DNA probes for evidence of centromeric DNA in estradiol induced micronuclei showed 70% contained centromeric sequences. This supports aneugenic activity and mitotic interference as the mode of action of E2 (Schuler *et al.*, 1993).

6.2.3 Regulatory View for Aneugenic Substances

The committee on "Mutagenicity of Chemicals in Food, Consumer Products and the Environment" (COM), an independent advisory committee of the United Kingdom Department of Health, concluded that it is reasonable to assume that aneuploidy-inducing chemicals have a

threshold of action (Parry *et al.*, 1994). This has been supported by experiments of Elhajouji *et al.* (1997) who demonstrated thresholds for induction of micronuclei after *in vitro* exposure of human lymphocytes to several well characterized aneugenic chemicals.

6.2.4 Discussion

For phenolphthalein, the most compelling question that must be answered with respect to potential genotoxicity is the source of micronucleus induction activity *in vivo*. With clear evidence of absence of clastogenic activity of phenolphthalein, (*i.e.*, the SCGE test results) the mode of action for micronucleus production cannot be concluded to be by DNA damage. The evidence supports an aneugenic mechanism. Dunnick *et al.* (1997) pointed out that phenolphthalein is weakly estrogenic, and binds to the estrogen receptor in receptor binding assays. The literature clearly shows that estrogens including 17β-estradiol and DES both induce micronuclei containing a significantly elevated frequency of kinetochore and centromere positive elements. Analysis of phenolphthalein induced micronuclei using CREST sera revealed 73% of the MN positive cells analyzed reacted with the kinetochore-specific antibodies (Tice and Furedi-Machacek, 1997). These kinetochore-rich micronuclei represent evidence for an aneugenic mode of action for phenolphthalein, in argument with the published examples of anuegens that also induce micronuclei.

Both DES and 17β-estradiol are listed (NIEHS, 1997) as hormones understudy "to evaluate alternative models", including the *p53+/-* mouse. Other classes of chemicals in this listing include 3 "human carcinogens that are genotoxic" an "immunosuppressant human carcinogen", 5 "rodent carcinogens that are putative human non-carcinogens (based on epidemiology", 7 "rodent carcinogens that are putative human non-carcinogens (based on mechanism)", and 3 "non-carcinogens. This discussion emphasizes the point that the *p53+/-* test system remains an experimental research tool, intended to confirm bioassay results for genotoxic carcinogens. Additional testing with known or suspected aneugens also should be carried out. Results of exposures to chemicals with aneugenic potential may produce responses that cannot be distinguished from the clearly genotoxic carcinogens tested to date (Tennant *et al.*, 1996). Such information is critical to this discussion of results in the *p53+/-* mice that are particularly sensitive to mutagenic carcinogens, since the effects of increasing the

frequency of non-disjunction, as with phenolphthalein, could be misinterpreted as a genotoxic response.

6.3 Conclusion

We believe the experimental evidence shows that phenolphthalein is not clastogenic, and therefore conclude that it induces thymic lymphomas and micronuclei in the *p53* transgenic mouse by a non-genotoxic mechanism.

A major goal of the more recent NTP work on phenolphthalein with the *p53*+/- experimental system was to confirm the results of the NTP (1996) mouse chronic bioassay, particularly to show that the observed ovarian tumors arose by a genotoxic mechanism. This was the reason for using female mice only. The facts that ovarian tumors were not found and that no DNA damage was found in the sensitive SCGE assay, provide very strong evidence that phenolphthalein is not genotoxic. Nonetheless, as often said, it is difficult to prove a negative.

Finally, it is important to note that research with the transgenic *p53*+/- mouse is progressing rapidly with the hope that it will prove to be a valuable research and decision-making tool once it is more fully understood. It is the position of this report that the data from the *p53*+/- mice show that phenolphthalein induces thymic lymphomas, but only by a non-genotoxic mechanism that has been described in detail above. For a regulatory agency to use these recent research data to label phenolphthalein as a genotoxic rodent carcinogen, therefore, would be a mistake. Furthermore, such a conclusion would set an unfortunate scientific precedent, and this should be of concern to the developers, researchers, and users of the *p53*+/- transgenic mouse system, as well as for those who will need to interpret its results.

It may be important to comment also on the evidence that shows phenolphthalein to have weak estrogenic activity and aneugenic properties. These properties help to explain some of the high dose tumorigenic effects in the mouse lifetime study but have no relevance to adults who take this medication occasionally, or even regularly, at much lower doses that in the chronic mouse bioassay (NTP, 1996).

The dosage question is another important point to raise in relation to the interpretation of the results from the experimental *p53+/-* transgenic system. This test may provide a suitable means to confirm bioassay results, or to explore carcinogenic mechanism. The application of exposure data from tumor suppressor deficient rodents in quantitative human risk assessment will require further extensive investigation. The test system is designed to be sensitive, and whatever mechanistic relevance it may achieve with additional research may prove to be extremely valuable in a qualitative sense. To use *p53+/-* test results quantitatively, however, for extrapolation to the human population, is not scientifically defensible at this time. Again, therefore, regulatory decisions that depend on dose levels in the sensitized *p53+/-* transgenic mice would be premature at best and would set an unfortunate precedent.

7.0 REFERENCES

Adler, S.S., and Trobaugh, F.E. 1978. Effects of estrogen on erythropoiesis and granuloid progenitor cell (CFU-C) proliferation in mice. J Lab Clin Med 91(6):960-968.

Alden C.L., and Frith, C.H. 1991. Urinary system. In: Haschek, W.M., and Rousseaux, C.G. (Eds), Handbook of Toxicologic Pathology. Academic Press, Inc., Toronto.

Bitman, J., and Cecil, H.C. 1970. Estrogenic activity of DDT analogs and polychlorinated biphenyls. J Agric Food Chem 18:1108-1112.

Bonin, A.M., Farquharson, J.B., and Baker, R.S.U. 1981. Mutagenicity of arylmethane dyes in *Salmonella*. Mutat Res 89:21-34.

CanTox. 1997. (As Appendix A). Discussion of New Data Related to Phenolphthalein Presented at the April 30, 1997 Meeting of the Carcinogenicity Assessment Committee of the U.S. Food and Drug Administration. Final Report. Prepared for. Novartis Consumer Health Corporation, Summit, NJ 07901-1312.

Capen, C.C., DeLellis, R.A., and Yarrington, J.T. 1991. III. Adrenal Medulla. In: Haschek, W.M., and Rousseaux, C.G. (Eds.) Handbook of Toxicologic Pathology. pp. 692-697.

Cheng, L. 1980. Pheochromocytoma in rats: Incidence, etiology, morphology and functional activity. J Environ Pathol Toxicol 4:219-228.

Coupland, R.E. 1969. The Natural History of the Chromaffin Cell, Longmans Green Ltd., Edinburgh. Cited In: Tischler *et al.*, 1989.

Coupland, R.E., and Tomlinson, A. 1989. The development and maturation of adrenal medullary chromaffin cells of the rat *in vivo*. A descriptive and quantitative study. Int J Dev Neurosci 7:419438. Cited In: Tischler and Coupland, 1994.

Davies, T.S., and Monro, A. 1995. Marketed pharmaceuticals reported to be carcinogenic in rodents. J Am Coll Toxicol 14(2):90-107.

Dietz, D.D., Elwell, M.R., Chapin, R.E., Shelby, M.D., Thompson, M.B., Filler, R., and Stedham, M.A. 1992. Subchronic (13-week) toxicity studies of oral phenolphthalein in Fischer 344 rats and B6C3F$_1$ mice. Fund Appl Toxicol 18(1):48-58.

Dunnick, J.K., Hardisty, J.F., Herbert, R.A., Seeley, J.C., Furedi-Machacek, E.M., Foley, J.F., Lacks, G.D., Stasiecwicz, S., and French, J.E. 1997. Phenolphthalein rapidly induces malignant hematopoietic tumors and loss of heterozygosity in the *p53* wildtype allele in heterozygous *p53* deficient (+/-) mice. Submitted for publication.

Duprat, P., Snell, K.C., and Hollander, C.F. 1990. Tumours of the Adrenal Gland. In: Turusov, V., and Mohr, U. (Eds.) Pathology of Tumours in Laboratory Animals. Volume 1. Tumours of the Rat. Second Edition. International Agency for Research on Cancer (WHO), International Life Sciences Institute. Lyon, No. 99. pp. 573-581.

Elhajouji, A., Tibaldi, F., and Kirsch-Volders, M. 1997. Indication for thresholds of chromosome non-disjunction versus chromosome lagging induced by spindle inhibitors *in vitro* in human lymphocytes. Mutagenesis 12:133-140.

Fairbairn, D.W., Olive, P.L., and O'Neill, K.L. 1995. The comet assay: A comprehensive review. Mut Res 339:37-59.

FDA. 1994. International Conference on Harmonisation; Dose Selection for Carcinogenicity Studies of Pharmaceuticals (Adopted October 1994). Food and Drug Administration. Federal Register 59(40):9752-9755.

Forsberg, J.-G. 1984. Short-term and long-term effects of estrogen on lymphoid tissues and lymphoid cells with some remarks on the significance for carcinogenicity. Arch Toxicol 55:79-90.

Fried, W., Tichler, T., Dennenberg, I., Barone, J., and Wang, F. 1974. Effects of estrogens on hematopoietic stem cells and on hematopoiesis of mice. J Lab Clin Med 83:807-815.

Frith, C.H., Davis, M.T., Zolotor, L.A., and Townsend, J.W. 1980. Histiocytic lymphoma in the mouse. Leukemia Res 4(6):651-662.

Fujita, H., Mizuo, A., and Hiraga, K. 1976. Mutagenicity of dyes in the microbiol system. Annu Rep Tokyo Metr Res Lab PH 27:153-158. Cited In: NTP, 1996.

Galloway, S.M., Bloom, A.D., Resnick, M., Margolin, B.H., Nakamura, F., Archer, P., and Zeiger, E. 1985. Development of a standard protocol for *in vitro* cytogenetic testing with chinese hamster ovary cells: Comparison of results for 22 compounds in two laboratories. Environ Mutagen 7:1-51.

Galloway, S.M., Armstrong, M.J., Reuben, S., Colman, S., Brown, B., Cannon, C., Bloom, A.D., Nakamura, F., Ahmed, M., Duk, S., Rimpo, J., Margolin, B.H., Resnick, M.A., Anderson, B., and Zeiger, E. 1987. Chromosome aberrations and sister chromatid exchanges in chinese hamster ovary cells: Evaluations of 108 Chemicals. Environ Mole Mutagen 10:1-175.

Griffin, R.J., Godfrey, V.B., and Burka, L.T. 1997. Metabolism and disposition of phenolthalein in male and female F344 and B6C3F1 mice. Submitted manuscript.

Hamlin, M.H., and Banas, D.A. 1990. Adrenal gland. In: Boorman, G.A., Eustis, S.L., Elwell, M.R., Montgomery, C.A. (Jr.), and MacKenzie, W.F. (Eds.) Pathology of the Fischer Rat: Reference and Atlas. Academic Press, New York, NY, pp. 501-518.

Hard, G.C. 1990. Tumors of the Kidney, Renal Pelvis, and Ureter. In: Pathology of Tumors in Laboratory Animals. Volume 1 - Tumors of the Rat, Lyon, France: International Agency for Research on Cancer. International Life Science Institute. IARC Scientific Publications No. 99, 301-324.

Hard, G.C., and Snowden, R.T. 1991. Hyaline droplet accumulation in rodent kidney proximal tubules: An association with histiocytic sarcoma. Toxicol Pathol 19(2):88-97.

Highman, B., Roth, S.I., and Greenman, D.L. 1981. Osseous changes and osteosarcomas in mice continuously fed diets containing diethylstilbestrol or 17B-estadiol. J Natl Cancer Inst 67:653-662.

IARC. 1979. IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans. Sex Hormones (II). Volume 21. International Agency for Research on Cancer, Lyon, France.

Kada, T., Tutikawa, K., and Sadaie, Y. 1972. *In vitro* and host-mediated "rec-assay" procedures for screening chemical mutagens; and phloxine, a mutagenic red dye detected. Mutat Res 16:165-174.

Kerckaert, G.A., Brauninger, R., LeBoef, R.A., and Isfort, R.J. 1996. Use of the Syrian hamster embryo cell transformation assay for carcinogenicity prediction of chemicals currently being tested by the National Toxicology Program in rodent assays. Environ Health Perspect 104(5):1075-1084.

Luster, M.I., Hayes, H.T., Korach, K., Tucker, A.N., Dean, J.H., Greenlee, W.F., and Boorman, G.A. 1984. Estrogen immunosuppression is regulated through estrogenic responses in the thymus. J Immunol 133(1):110-116.

Luster, M.I., Pfeifer, R.W., and Tucker, A.N. 1985. The Immunotoxicity of Natural and Environmental Estrogens. In: Dean, J.H., Luster, M.I., Munson, A.E., and Amos, H. (Eds.), Immunotoxicology and Immunopharmacology, Target Organ Toxicology Series, Raven Press, New York, pp. 315-326.

Lynch, B.S., Tischler, A.S., Capen, C., Munro, I.C., McGirr, L.M., and McClain, R.M. 1996. Low digestible carbohydrates (polyols and lactose): Significance of adrenal medullary proliferative lesions in the rat. Regul Toxicol Pharmacol 23(3):256-297.

Manger, W.M., Gifford, R.W. (Jr.), and Hoffman, B.B. 1985. Pheochromocytoma: A clinical and experimental overview. Curr Prob Cancer 9:1-89.

Marrazzini A., Betti, C., Bernacchi, F., Barrai, I., and Barale, R. 1994. Micronucleus test metaphase analyses in mice exposed to known and suspected spindle poisons. Mutagenesis 9:505-515.

Minno, A.M., Bennett, W.A., and Kvale, W.F. 1954. Pheochromocytoma: A study of 15 cases diagnosed at autopsy. N Engl J Med 251:959-965. Cited In: Manger *et al.*, 1985.

Montgomery, C.A., Jr., and Seely, J.C. 1990. Kidney. In: Boorman, G.A., Eustis, S.L., Elwell, M.R., Montgomery, C.A., Jr., and MacKenzie, W.F. (Eds.), Pathology of the Fischer Rat: Reference and Atlas. Academic Press, New York, London, Toronto, pp.127-153.

Mortalmans, K., Haworth, S., Lawlor, T., Speck, W., Tainer, B., and Zeiger, E. 1986. *Salmonella* mutagenicity tests: II. Results from testing of 270 chemicals. Environ Mutagen 8(Suppl. 7):1-119.

NIEHS. 1997. Testing Information and Study Results. National Institute Environmental Health Sciences. NTP Testing Information, Website.

Nestmann, E.R., Bryant, D.W., Carr, C.J., Fennell, T.T., Gallagher, J.E., Gorelick, N.J., and Swenberg, J.A. 1996. Toxicological significance of DNA adducts: Summary of discussions with an Expert Panel. Regul Toxicol Pharmacol 24:9-18.

Nieto, A., Garcia, C., and Lopez de Haro, M.S. 1990. *In vivo* estrogenic and anti-estrogenic activity of phenolphthalein and derivative compounds. Biochem Int 21:305-311.

NTP. 1996. Toxicology and Carcinogenesis Studies of Phenolphthalein in F344/N Rats and B6C3F1 Mice. National Toxicology program, Technical Report Series No. 465. U.S. Department of Health and Human Services.

Parry, J.M., Fielder, R.J., and McDonald, A. 1994. Thresholds for aneuploidy-inducing chemicals. Mutagenesis 9(6):503-504.

Pattengale, P.K., and Frith, C.H. 1986. Contributions of recent research to the classification of spontaneous lymphoid cell neoplasms in mice. CRC Crit Rev Toxicol 16:185-212. Cited In: Hard and Snowden, 1991.

PDR. 1996. Physicians' Desk Reference. Medical Economics, Montvale, NJ.

Phillips, D.H. 1997. Detection of DNA modifications by the ^{32}P-postlabelling assay. Mutat Res 378:1-12.

Pollard, M., and Luckert, P.H. 1989. Spontaneous diseases in aging Lobund-Wistar rats. In: Snyder, D.L. (Ed.) Dietary Restriction and Aging. Alan R. Liss Inc., New York, NY. Symposium on the Effects of Dietary Restriction on Aging and Disease in Germfree and Conventional Lobund-Wistar Rats - Proceedings, Mar. 27- 29, 1988, Notre Dame, IN. Vol. 287:51-60.

Ravdin, P.M., van Beurden, M., and Jordan, V.C. 1987. Estrogenic effects of phenolphthalein on human breast cancer cells *in vitro*. Breast Cancer Res Treat 9:151-154.

Roe, F.J.C. 1993. Mineral Metabolism and Chemical Toxicity. In: Parke, D.V., Ioannides, C., and Walker, R. (Eds.), Food, Nutrition and Chemical Toxicity. Smith-Gordon and Company Ltd., London, UK:Nishimura and Company Ltd., Niigata-Shi, Japan, pp. 91-104.

Roe, F.J.C. 1996. Phenolphthalein: Opinion on the findings in laboratory tests for toxicity and carcinogenicity. February 8, 1996.

Roe, F.J.C., and Bär, A. 1985. Enzootic and epizootic adrenal medullary proliferative disease of rats: Influence of dietary factors which affect calcium absorption. Human Toxicol 4(1):27-52.

Selim, S., and Luke, C. 1992. Excretion Balance and Pharmacokinetic Study of ^{14}C-Labeled Phenolphthalein after a Single Oral Dose in Human Volunteers. Biological Test Center, 2525 McGraw Avenue PO Box 19791, Irvine, CA 92713-9791. BTC Study No. P0392002. Sponsor: Nonprescription Drug Manufacturers Association, 1150 Connecticut Avenue NW, Washington DC, 20032. November 24, 1992.

Sass, B., and Montali, R.J. 1980. Spontaneous fibro-osseous lesions in aging female mice. Lab Animal Sci 30:907-909.

Schardein, J.L. *et al.*, 1968. Spontaneous tumors in Holtzman Source rats of various ages. Pathol Vet 5:238-252. Cited In: Thompson *et al.*, 1981.

Schiffman, D., and De Boni, U. 1991. Dislocation of chromatin elements in prophase induced by diethylstilbestrol: a novel mechanism by which micronuclei can arise. Mutat Res 246:113-122.

Schnitzler, R., Foth, J., Degen, G., and Metzler, M. 1994. Induction of micronuclei by stilbene-type and steroidal estrogens in Syrian hamster embryo and ovine seminal vesicle cells in vitro. Mutat Res 311:85-93.

Schuler, M., Zankl, H., and Metzler, M. 1993. Comparison of diethylstilbestrol and 17β-estradiol for the induction of mitotic arrest and micronucleation in human chronic villi cells. J Cancer Res Clin Oncol 119:Suppl 2, 55.

Schuler, M., Rupa, D.S., and Eastmond, D.A. 1997. A critical evaluation of centromeric labeling to distinguish micronuclei induced chromosomal loss and breakage in vitro. Mutat Res 392:81-95.

Sgura, A., Antoccia, A., Ramirez, M.J., Marcos, R., Tanzarella, C., and Degrassi F. 1997. Micronuclei, centromere-positive micronuclei and chromosome nondisjunction in cytokinesis blocked human lymphocytes following mitomycin C or vincristine treatment. Mutat Res 392:97-107.

Sher, S.P., Jensen, R.D., and Bokelman, D.L. 1982. Spontaneous tumors in control F344 and Charles River-CD rats and Charles River-CD-1 and B6C3F1 mice. Toxicol Lett 11:103-110.

Sipe, H.J., Corbett, J.T., and Mason, R.P. 1997. *In vitro* free radical metabolism of phenolphthalein by peroxidases. Drug Metab Dispos 25:(4):468-480.

Stopper, H., Eckert, I., Schiffmann, D., Spencer, D.L., and Caspary, W.J. 1994. Is micronucleus induction by aneugens an early event leading to mutagenesis? Mutagenesis 9:411-416.

Strandberg, J.D. 1983. Hyperplasia: adrenal medulla, rat and pheochromocytoma adrenal medulla, rat. In: Jones, T.C., Mohr, U., and Hunt, R.D., (Eds.) Endocrine System. Springer-Verlag, New York, NY. Monographs on Pathology of Laboratory Animals Copy Chapter.

Symington, T.. 1969. Functional Pathology of the Human Adrenal Gland. E. & S. Livingstone, Ltd., London, England, pp. 215-324. Cited In: Thompson *et al.*, 1981.

Tennant, R.W., Spalding, J., and French, J.E. 1996. Evaluation of transgenic mouse bioassays for identifying carcinogens and noncarcinogens. Mutat Res 365:119-127.

Thompson, S.W., Rac, V.S., Semonick, D.E., Antonchak, B., Spaet, R.H., and Schellhammer, L.E. (Jr.) 1981. The Adrenal Medulla of Rats: Comparative Physiology, Histology, and Pathology. Charles C. Thomas, Springfield, IL, pp. 102.

Tice, R.R., and Furedi-Machacek, E.M. 1997. Tumorigenicity Studies of Dietary Phenolphthalein (CAS No. 77-09-8) in TSG-*p53*™Transgenic Female Mice. Integrated Laboratory Systems (ILS), Project No. T051; MSE No. 49-95.

Tischler, A.S. 1992. Proliferative responses to chromaffin cell mitogens by adult rats and human chromaffin cells *in vitro*. Lab Invest 66:34A.

Tischler, A.S., and DeLellis, R.A. 1988. The rat adrenal medulla II. Proliferative lesions. J Am Coll Toxicol 7(1):23-41.

Tischler, A.S., and Riseberg, J. 1993. Different responses to mitogenic agents by adult rat and human chromaffin cells *in vitro*. Endocr Pathol 4(1):15-19.

Tischler, A.S., and Coupland, R.E. 1994. Changes in Structure and Function of the Adrenal Medulla. In: Mohr, U., Dungworth, D.L., and Capen, C.C. (Eds.) Pathobiology of the Aging Rat, Volume 2, pp. 245-268.

Tischler, A.S., DeLellis, R.A., Perlman, R.L., Allen, J.M., Costopoulos, D., Lee, Y.C., Nunnemacher, G., Wolfe, H.J., and Bloom, S.R. 1985. Spontaneous proliferative lesions of the adrenal medulla in aging Long- Evans rats: Comparison to PC12 cells, small granule-containing cells, and human adrenal medullary hyperplasia. Lab Invest 53:486-498.

Tischler, A.S., Ruzicka, L.A., Donahue, S.R., and DeLellis, R.A. 1989. Chromaffin cell proliferation in the adult rat adrenal medulla. Int J Develop Neurosci 7:439-448.

Tischler, A.S., Powers, J.F., Downing, J.C., Riseberg, J.C., Shahsavari, M., Ziar, J., and McClain, M. 1996. Vitamin D_3, lactose, and xylitol stimulate chromaffin cell proliferation in the rat adrenal medulla. Toxicol Appl Pharmacol 140(1):115-123.

Tsutsui, T., Tamura, Y., Yagi, E., Hasegawa, K., Tanaka, Y., Someya, T., Hamaguchi, F., Yamamoto, H., and Barrett, J.C 1997. Cell transforming activity and genotoxicity of phenolthalein in cultured Syrian hamster embryo cells. Submitted manuscript.

Tucker, R.W., and Barrett, J.C. 1986. Decreased numbers of spindle and cytoplasmic microtubules in hamster embryo cells treated with a carcinogen, diethylstilbestrol. Cancer Res 46:2088-2095.

Van Goethem, F., Lison, D., and Kirsch-Volders, M. 1997. Comparative evaluation of the in vitro micronucleus test and the alkaline single cell gel electrophoresis assay for the detection of DNA damaging agents: genotoxic effects of cobalt powder, tungsten carbide and cobalt-tungsten carbide. Mutat Res 392:31-43.

Vig, B.K., and Swearngin, S.E. 1986. Sequence of centromere separation. Kinetochore formation in induced laggards and micronuclei. Mutagenesis 1:461-465.

von Schlegel, G.G. 1960. Neurofibromatose Recklinghausen und Phäochromocytom. Schweiz Med Wochenshr 90:31-39. Cited In: Manger *et al.*, 1985.

Ward, J.M., and Sheldon, W. 1993. Expression of mononuclear phagocyte antigens in histiocytic sarcoma of mice. Vet Pathol 36:560-565.

Witt, K.L., Gulati, D.K., Kaur, P., and Shelby, M.D. 1995. Phenolthalein: Induction of micronucleated erythrocytes in mice. Mut Res 341:151-160.

APPENDIX A:

DISCUSSION OF NEW DATA RELATED TO PHENOLPHTHALEIN PRESENTED AT THE APRIL 30, 1997 MEETING OF THE CARCINOGENICITY ASSESSMENT COMMITTEE OF THE U.S. FOOD AND DRUG ADMINISTRATION

CANTOX INC.

Consultants in Toxicology
Health and Environmental Sciences

DISCUSSION OF NEW DATA RELATED TO PHENOLPHTHALEIN PRESENTED AT THE APRIL 30, 1997 MEETING OF THE CARCINOGENICITY ASSESSMENT COMMITTEE OF THE U.S. FOOD AND DRUG ADMINISTRATION

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1.0 TRANSGENIC MODELS FOR MUTAGENESIS AND CARCINOGENESIS

A transgenic animal is one in which the DNA has been altered in a heritable manner, with foreign DNA sequences or altered endogenous DNA sequences that are inserted into the genome of the zygotes or embryos. The sources of these sequences are varied (Mirsalis *et al.*, 1994)

Current transgenic animals for mutagenesis and carcinogenesis have developed along two conceptual lines:

i) those designed to provide a model to quantitatively measure *in vivo* mutagenesis; and,

ii) those designed to evaluate the role of various endogenous genes, including oncogenes and tumor suppressor genes in the carcinogenic process.

1.1 *In vivo* Mutagenesis

DNA sequences introduced encode for proteins of the *lac* operon in *E. coli* These include the *lac* repressor binding sequence or *lac* I and the gene for β-galactosidase or *lac* Z.

Treated mice or rats that carry the inserted foreign DNA in each of their cells are sacrificed following exposure, and the inserted DNA of known sequence is recovered. Analysis of the recovered sequences indicates the presence of mutations, and the mutagenic potency can be determined through comparison of mutant frequencies found in untreated control transgenic animals (Mirsalis *et al.*, 1995). The inserted genes can be recovered by various molecular techniques.

Transgenic animal models developed to assess the role of altered function of specific genes involved in carcinogenesis contain constructs of over-expressed, defective, or inactive forms of endogenous genes.

These genes may be involved in various aspects of cell cycle regulation, cell-to-cell communication, and cell differentiation and proliferation such as oncogenes including v-Ha-*ras* and the *p53* tumor suppressor gene.

Transgenic systems have been developed which target endogenous genes including various oncogenes and tumor suppressor genes.

The *p53* gene is the most studied tumor suppressor gene (Harris, 1996). Inactivation of the *p53* gene product also has been demonstrated to occur in a number of human tumors (Donehower, 1996). It has been estimated that of the approximately 6.5×10^6 annually reported cancer cases worldwide, thirty-seven percent have tumors that contain a *p53* mutation. Tumor suppressor genes maintain tissue homeostasis through control of cellular proliferation, terminal differentiation and programmed cell death (apoptosis) (Harris, 1996). Tumor types historically known to contain a mutant *p53* gene are listed in Table 1 along with their estimated expected frequency of appearance in the population.

Table 1 Frequency of New Cancer Cases with Mutant *p53* Suppressor Gene[a]

Tumor type (developed countries)	Estimated annual frequency ($\times 10^{-3}$)	Estimated frequency of *p53* mutation ($\times 10^{-3}$)
Stomach	333	137
Lung	455	255
Breast	348	84
Colon-rectum	389	191
Cervix	96	8
Mouth-pharynx	108	39
Esophagus	57	26
Liver	60	15
Lymphoma	116	14
Prostate	177	62
Bladder	148	50
Leukemia	83	9
Corpus uteri	104	28
Ovary	70	31
Pancreas	90	41
Larynx	53	23

[a] Source: C.C. Harris (1996).

The *p53* gene provides a biochemical component central to human carcinogenesis. Mutations of this gene by alteration though missense mutation or through the introduction of nonsense or frameshift mutations provides a selective advantage for clonal expansion of preneoplastic and neoplastic cells (Harris, 1996).

The current understanding of wildtype *p53* function provides insight for the role of mutant *p53* in tumorigenesis. Wildtype *p53* is a negative growth regulator. In other words, in "normal" individuals, the product of the *p53* gene regulates the proliferation of the cells keeping uncontrolled cell growth and cell division in check. In normal cells, wildtype *p53* protein is greatly increased following treatment with ionizing radiation and mutagens that directly or indirectly induce DNA strand breaks. In cells which lack functional *p53* genes, the introduction of a source of *p53* protein can arrest cells in late G1 of the cell cycle or initiate apoptosis leading to a programmed cell death. The introduction of a checkpoint in the cell cycle at G1 can be responsible for arrest of the cell cycle that in turn allows DNA repair systems to act and remove damage prior to entry into the DNA synthetic, or S phase (Donehower, 1996).

p53 has been termed the "guardian of the genome" (Lane, 1992). One of the inferences of this hypothesis is that cells that lose *p53* would fail to arrest at G1 or undergo apoptosis after suffering DNA damage. Progression of DNA synthesis in the presence of a damaged DNA template would lead to genetic instability. This instability could be reflected in increased aneuploidy and/or chromosomal aberrations due to interference with mitotic spindle function and chromosomal segregation. Other evidence of genetic instability can be found in chromosomal alterations such as translocations, amplification or deletion, and point mutation. Mouse data from investigations of mammary tumor progression in the absence of functional *p53* provide evidence of selective loss of specific chromosomes or chromosomal segments. This work has suggested that the acceleration of tumor progression may be achieved because of the role of *p53* loss in promotion of genomic instability (Donehower, 1996).

There are different variants or classes of *p53* mutations, indicating that partially functional gene products are produced. These mutants with altered transcriptional activity possess a gain-of-function activity and, therefore, a poorer prognosis when compared to tumors with no *p53* at all. This can be demonstrated in mice modified by the insertion of a *p53* mutant transgene *Val-135* into animals with one or two endogenous copies of wildtype *p53*. Such

animals show accelerated spontaneous tumor development *in vivo*, while presence of the gene promotes cell growth *in vitro* (Harvey *et al.*, 1995). Another consequence of the mutation that produces altered up regulation of *p53* in transcriptionally functional mutants is a significant change in the spectrum of spontaneous tumor sites found in heterozygous *p53+/-* mice carrying the transgene. Harvey *et al.*(1995) reported that animals with the transcriptionally active transgene had increased numbers of lung adenocarcinomas and lymphomas, but decreased numbers of osteosarcomas and soft tissue sarcomas when compared to mice without the extra mutation (Table 2).

Table 2 Spontaneous Tumors in *p53*± Heterozygous or *p53*-/- nullizygous Mice and The Effect of a Partially Active *p53 Val 135* Transgene[a]

Tumor Type	*p53*± (%)	*p53* ± *135* (%)	*p53*-/- (%)	*p53*-/- *Val 135* (%)
Lymphomas	16 (27)	14 (40)	35 (83)	17 (89)
Osteosarcomas	14 (24)	3 (9)	2 (5)	
Hemangiosarcomas	7 (12)	4 (11)		1 (5)
Lung adenocarcinoma	1 (2)	7 (20)	1 (2)	
Rhabdomysarcomas	4 (7)	1 (3)	1 (2)	1 (5)
Undifferentiated sarcomas	4 (7)	1 (3)	1 (2)	
Meningial sarcomas	3 (5)			
Leiomyosarcomas	2 (3)	1 (3)		
Squamous cell carcinomas	2 (3)	1 (3)		
Other	7 (13)	3 (8)	2 (6)	

Other includes Liposarcoma, Fibrosarcoma, Pancreatic islet cell carcinoma, Papillary carcinoma, Teratocarcinoma, Perianal adenosarcoma, Mammary adenocarcinoma, Intestinal adenocarcinoma, and Eye adenocarcinoma.

[a] Table from Harvey *et al.*, 1995.

1.2 The *p53*-Deficient or "Knockout" Mouse

There are two variants of this model, one in which mice of either the C57Bl/6 or Sv/129 strain contain a single wild-type *p53* allele and a null allele (*i.e.*, heterozygous, ±) and one in which these strains contain two null alleles (*i.e.*, nullizygous, *p53-/-*).

p53-Deficient mice are susceptible to spontaneous and to chemically-induced tumor development. Tumors develop faster and at a higher incidence than in wild-type mice. Mice with an inactive *p53* gene (heterozygous) have a low background tumor incidence for up to 12 months of age. This is in contrast with the nullizygous mice that have a higher rate of spontaneous tumors leading to less than 10% survival by four months.

The heterozygous *p53*-deficient mouse can detect in 24 weeks carcinogenic endpoints resulting from chemical exposure at the same doses as used in 2-year bioassay.

The loss of one allele reduces the number of mutational events required for the complete loss of *p53* function. Also, *p53* appears to mediate cell cycle arrest following certain types of DNA damaging events.

The *p53*-deficient mouse model **is responsive to genotoxic, but not non-genotoxic, carcinogens** (*i.e.*, non-genotoxic carcinogens do not result in mutations in the active allele, and thus do not exacerbate the *p53*-deficient state). Tennant *et al.* (1995) reported results for *p53+/-* mice in two trans-species carcinogens (*i.e.* induce tumors in both rats and mice) that had been examined in the NTP two year bioassay and that were also known to be genotoxic *in vitro* assays: *p*-cresidine and 4-vinyl-1-cyclohexane diepoxide (Table 3). These authors also examined the response of male and female mice to the nongenotoxic, single species carcinogens *N*-methyloacrylamide and resperine. The bacterial mutagen *p*-anisidine was also examined. This chemical is positive in the *Salmonella* assay, but failed to produce a carcinogenic response in either species in the NTP bioassay.

The results, reproduced in Table 3, show a good correlation between the results of the two year studies supported by the NTP and the results observed by Tennant *et al.* (1995) with *p53+/-*. Chemicals that were not both genotoxic and carcinogenic did not produce tumors. Furthermore, the type and site of tumor development in the *p53+/-* was similar to that found in

the longer NTP study. On the basis of this comparison, it would appear that the *p53+/-* mouse demonstrates a very high specificity and sensitivity for genotoxic carcinogens.

Harvey *et al.* (1993) have reported results for tumor type and frequency induced by feeding dimethylnitrosamine (DMN) in drinking water to *p53+/-* and *p53+/+* mice. This carcinogen is a potent inducer of hemangiosarcomas as well as lung tumors. No heterozygous mice survived beyond 39 weeks, and had a mean survival time of 29 weeks. The *p53+/+* mice survived 62 weeks of exposure and had a mean survival time of 42 weeks. Histological examination of the DMN treated animals of either genotype showed evidence of multiple hemangiosarcomas. A minority of the heterozygous and *p53+/+* animals had lung lesions.

Kemp *et al.* (1994) have reported that time to tumor following whole body γ irradiation (4 Gy) of *p53+/-* mice is significantly reduced compared with untreated *p53+/-* animals. None of the irradiated *p53+/+* mice developed tumors. The median age to tumor development in the irradiated heterozygous mice was 40 weeks, considerably reduced from the >70 weeks found in *p53+/-* mice. The spectrum of tumor types was similar for the irradiated and unirradiated *p53+/-* mice. The etiology of the spontaneous mutants is unknown, but the similarity of spectrum with irradiated *p53+/-* suggests that irradiation accelerated the process of tumor development in some unspecified manner. It was speculated that ionizing low linear energy transfer radiation (LET) generates oxygen radicals in tissues, resulting in DNA damage (Kemp *et al.*, 1994).

In summary, there are several reports in the literature that support the hypothesis that chemical carcinogens that produce a specific type of tumor in B6C3F1 mice will reproduce similar tumors in *p53+/-* mice heterozygous for the *p53* allele. Furthermore, it would appear that time to tumor is accelerated in these strains when compared to *p53+/+* animals. An exception to this is with exposure to γ radiation which unaccountably produces the same spectrum of tumor sites found spontaneously in the population of heterozygotes. It is not clear whether production of oxygen radicals in tissues in response to γ radiation produces any unique tumor types, or whether this damage simply accelerates a spontaneous process.

Table 3 Target Organ Tumor Incidence in the 24-week Study C57BL/6 *p53*-Deficient Mice and NTP 103-week Study in B6C3F1 Hybrid Mice[a]

Chemical (route: target)	Dose 24 wk	C57BL/6 *p53* ♂	C57BL/6 *p53* ♀	Wildtype ♂	Wildtype ♀	Dose 103 wk	Wildtype ♂	Wildtype ♀
4-vinyl-1-cyclohexene diepoxide (topical: skin) ***genotoxic***	0 mg	0/7	0/8	0/5	0/5	0 mg	0/50	0/50
	12.5 mg	2/7	0/8			5 mg	40/50	37/50
	25 mg	3/10	3/8	0/5	0/5	10 mg	43/50	43/50
p-Cresidine (diet: bladder) ***genotoxic***	0%	0/5	0/5	0/5	0/5	0%	0/50	0/50
	0.25%	4/7	0/8			0.25%	31/31	44/46
	0.5%	9/10	4/10	0/5	0/5	0.5%	40/42	41/46
p-Anisidine (diet: none) ***genotoxic in Salmonella***	0%	0/5	0/5	0/5	0/5	0%	non-carcinogen	
	0.225%	0/8	0/7			0.25%		
	0.45%	0/10	0/10	0/5	0/5	0.5%		
N-Methylolacrylamide (gavage: liver) ***non-genotoxic***	0 mg/kg	0/7	0/7	0/5	0/5	0 mg/kg	12/50	3/50
	25 mg/kg	0/7	0/7			25 mg/kg	17/50	4/50
	50 mg/kg	0/10	0/10	0/5	0/5	50 mg/kg	26/50	17/49
Resperine (diet: ♂ seminal vesicle; ♀ mammary gland) ***non-genotoxic***	0%	0/7	0/8	0/5	0/5	0%	0/50	0/50
	0.0005%	0/8	0/7			0.0005%	0/50	7/49
	0.001%	0/10	0/10	0/5	0/5	0.001%	0/50	7/48

[a] Source: Tennant *et al.*, 1995.

Advantages Presented by the TSG *p53*+/− ® Mouse for Detection of Genotoxic Carcinogens:

- mutagenesis is examined in the context of the whole animal *in vivo* where physiological and metabolic pathways, repair mechanisms and replication modes are functioning in concert;
- the assay is not tissue specific; thus mutation in a wide variety of tissues can be evaluated; and,
- the target for mutagenesis is an endogenous gene, and is small relative to the entire.

genome.

What was the rationale for proceeding with the phenolphthalein studies presented on April 30, 1997. The studies were designed as a comprehensive approach to the question of whether phenolphthalein is a genotoxic carcinogen and its mechanism of action. In our opinion the studies and data presented show some clear direction toward resolving this question, but many inconsistencies and important questions remain to be answered.

1. The studies consisted of two reports aimed at elucidating the metabolism, disposition and possible mode of activation of phenolphthalein in the rodent. These should lay the mechanism and groundwork for the next step which was to demonstrate that phenolphthalein acts as a genotoxic carcinogen.

2. There followed two reports on the genotoxicity of phenolphthalein *in vitro* and *in vivo*. The tests applied and described in the report of Tice *et al.* were designed to elucidate the mechanism of action of phenolphthalein through the use of several sensitive assays to show evidence of DNA damage from *in vivo* exposure to phenolphthalein. The work of Tsutsui *et al.* was meant to provide additional support through the use of *in vitro* assays using Syrian hamster embryo (SHE) cells to show that phenolphthalein is a clastogen, a mutagen and is capable of inducing cell transformation.

3. One report presents molecular biological data that analyze lymphoma DNA for the presence or absence of the wildtype allele of the *p53* gene in rodent tumors.

4. The main Integrated Laboratory Systems (ILS) study of the tumorigenicity of phenolphthalein in female *p53+/-* mice treated for a period of twenty-four weeks with various doses of phenolphthalein.

5. Finally, two epidemiological reports addressed the issue of evidence for increased cancer among the human population as a result of exposure to phenolphthalein containing laxatives. These are beyond the scope of this report and will not be addressed.

The reports will be taken in this order, since some of the evidence and data presented in the earlier reports will help to make clear the expected conclusions of succeeding presentations.

2.0 METABOLISM AND DISPOSITION

At ordinary dose levels, phenolphthalein is metabolized to its glucuronide conjugate in the intestine and the liver. It is excreted in the bile primarily as the glucuronide, with very small quantities excreted as the unmetabolized phenolphthalein (Sipe *et al.*, 1997). The rapid conjugation of phenolphthalein is in contrast to the duration of the laxative effect associated with this chemical. It has been proposed that this is largely due to the activity of bacterial glucuronidase in the intestine that leads to cleavage of the conjugated molecule and extensive enterohepatic recirculation of phenolphthalein. This view has been supported by data showing decreased enterohepatic circulation in rats treated with antibiotics to suppress intestinal microflora (Parker *et al.*, 1980). Sipe *et al.* (1997) have investigated the potential for the phenolic substituents of phenolphthalein to act as substrates for peroxidase, with the resultant release of superoxide radicals. The proposed mechanism would be through the production of phenoxyl radical metabolites that could react with glutathione (GSH) and NAD(P)H to regenerate the parent phenolphthalein and a radical such as GS· or NAD· . Either of these products would then produce superoxide. In this "futile metabolism", cyclic oxidation and reduction of phenolphthalein in combination with enterohepatic recirculation of phenolphthalein could lead to production of significant levels of superoxide. This is important mechanistically, since superoxide acts as a clastogen, and could be responsible for the production of single strand breaks in DNA.

Sipe *et al.* (1997) have established that phenolphthalein is a substrate for lactoperoxidase *in vitro*, and that oxygen is consumed and there is evidence of superoxide production. This oxygen consumption was sensitive to the presence of catalase and superoxide dismutase which effectively inhibited the production of $O_2\cdot^-$. Sipe *et al.* (1997) also found evidence that the glucuronide conjugate could undergo peroxidation to a limited extent. There was no direct evidence of free radical formation *in vivo* or evidence to support reaction with DNA to induce single strand breaks.

Griffin *et al.* (1997) did find evidence of a hydroxylated metabolite of phenolphthalein in female mouse tissues (these products were not found in male mice and were absent in the rat), but detected limited amounts in bone marrow. This is suggestive of production of hydroxyl radicals in the mouse. H_2O_2 would be a product of the peroxidase reaction proposed by Sipe

et al. (1997), and this could be a source of hydroxyl radical in the presence of reduced iron. Other findings of Griffin *et al.* (1997) showed rapid clearance of phenolphthalein metabolites and no appreciable evidence of concentration of metabolites in tissues of rats or mice. This leads to the conclusion that stable phenolphthalein derived DNA adducts are unlikely to be related to the carcinogenicity of phenolphthalein.

2.1 Summary

There is support for a free radical mechanism and production of $O_2^{\cdot -}$ from peroxidase mediated metabolism of phenolphthalein. There is some corroborating evidence of this from metabolites found only in female mice, but not in male mice or the rat. Thus, on the basis of *in vitro* evidence, there is possible support for a genotoxic mechanism related to single strand breaks in DNA. Though rats and mice have similar tissue concentrations of peroxidases, Griffin *et al.* (1997) only found direct evidence of hydroxylation of phenolphthalein in female mice. The results of the NTP bioassay of phenolphthalein in rats and mice showed evidence of tumor production in both species and both sexes. Therefore, it must be concluded that evidence of disposition and metabolism of phenolphthalein has not produced clear evidence of a mechanism for the presumed genotoxicity observed in other systems.

3.0 GENOTOXICITY *IN VITRO*

Results with phenolphthalein treatment of Syrian hamster embryo (SHE) cells *in vitro*.

Tsutsui *et al.* (1997) reported a series of results in a SHE cell system that showed evidence for cell transformation, chromosomal aberrations and mutation. Previously, Kerckaert *et al.* (1996) had found that phenolphthalein was positive for cell transformation in SHE cells. These authors found significant induction of cell transformation in the 60 to 80 μM range. Tsutsui *et al.* (1997) found significant induction at a somewhat lower range of doses (10 to 40 μM).

In a somewhat unusual step, Tsutsui *et al.* (1997) examined SHE cells for mutation at HPRT and Na^+/K^+ ATPase by selection for 6-thioguanine or ouabain resistance. Precedent for mutation induction in SHE cells was set by Tsutsui *et al.* (1984) who showed that amitrole, a

pesticide that produced negative or mixed responses in conventional mutation assays, was clearly positive in SHE cells. Amitole has been reported non-genotoxic in some forty genotoxicity assays in prokaryotes, three other tests for mutation in mammalian cells *in vitro*, and two tests for induction in micronuclei *in vivo*. The single positive report for evidence of genotoxic effect of amitrole was made by Tsutsui *et al.* (1984). SHE cells are genetically stable and capable of metabolizing many chemicals to their ultimate mutagenic form (Kerckaert *et al.*, 1997). However, they are a mixed population, and therefore present a nonuniform target for mutation assays. In the present report, phenolphthalein was not mutagenic at Na^+/K^+ ATPase, but was reported to give a positive response at HPRT. This is curious, since all doses produced mutation frequencies below the control value of less than 4×10^{-6} with the exception of the highest (40 μM = 5.6×10^{-6}). Since no data were presented to support the mutation frequency values, it is impossible to comment further.

Tsutsui *et al.* (1997) also examined SHE cells for chromosomal aberrations and found evidence of breaks, gaps and exchanges at the highest dose (40 μM), although the results were based on only 200 metaphases. Similar results were reported for aberrations in CHO cells with microsomal activation (NTP 465; Witt *et al.*, 1995). There was no evidence of aneuploidy induction at any of the doses tested.

Finally, Tsutsui *et al.* (1997) failed to show any evidence of DNA adducts in cells treated with phenolphthalein using a ^{32}P-postalbeling assay. This is not surprising, since Griffin *et al.* (1997) found some protein adducts, but no evidence that ^{14}C-phenolphthalein remained associated with tissues after *in vivo* exposures in rats and mice.

3.1 Summary

The conclusions in the paper submitted by Tsutsui *et al.* (1997) are given scant support in the form of actual data. The results that are discussed are only presented in summary format. In general, the conclusions that are drawn reiterate conclusions presented elsewhere and do not assist in improving the understanding of the mechanism of the observed rodent carcinogenicity of phenolphthalein.

4.0 GENOTOXICITY *IN VIVO*

The question of genotoxic mechanism for phenolphthalein has been most directly and comprehensively addressed by Tice *et al.* (1997). In conjunction with an *in vivo* study in female *p53+/-* they have applied a number of assays in an effort to elucidate mechanisms of DNA damage. These took several forms:

- micronucleus induction in polychromatic erythrocytes and normochromatic erythrocytes;
- single cell gel electrophoresis (comet) assay;
- kineticore analysis of micronuclei in peripheral erythrocytes; and,
- analysis of hemopoietic cells for evidence of apoptosis.

Phenolphthalein induces micronuclei in mice either by feeding or gavage studies at the relatively high doses ≥2000 mg/kg/day for two days, or after longer periods (14 weeks) at 120 mg/kg/day (Witt *et al.*, 1995). The work presented by Tice *et al.* (1997) support and extend these results using the *p53+/-* mouse model. After 39 days, significant numbers of micronuclei were found in both polychromatic erythrocytes (PCE) and normochromatic erythrocytes (NCE) at the two highest doses (3,000 ppm and 12,000 ppm). By 92 days, significant numbers of micronuclei were observed at all but the lowest administered dose (200 ppm). The actual doses in mg/kg/day varied. The daily intake was 37 ± 5.9 mg/kg (200 ppm), 71 ± 11.9 mg/kg (375 ppm), 146 ± 30.3 mg/kg (750 ppm), 569 ± 122.2 mg/kg (3,000) and 2074 ± 294.5 mg/kg (12,000 ppm).

The dose dependent increase in PCE with micronuclei suggests bone marrow or hemopoietic toxicity, since the transit time/lifetime of a PCE is 1-3 days, while NCEs remain in the bloodstream for five to six weeks. NCEs are considered evidence of integrated micronuclei induction over a protracted period of exposure. Micronuclei in PCE, on the other hand should reflect more current information relating to genotoxic effect.

Phenolphthalein has been identified as a genotoxic carcinogen in rodents. The mechanism of DNA damage produced by phenolphthalein remains to be determined. As outlined above, Sipe

et al. (1997) suggested that on the basis of *in vitro* analysis of phenolphthalein metabolism, one possible mechanism could be through the production of super oxide and single strand breaks. To test this hypothesis or to provide additional support for a genotoxic mechanism for phenolphthalein, Tice *et al.* (1997) collected blood from mice given various doses of phenolphthalein at several points in the study (day 39, 92, 137 and at termination). Each sample was examined by the single cell gel ectrophoresis (SCGE) assay.

Briefly, the SCGE assay provides a means of examining DNA from single cells to determine its state of fragmentation. For example, the examination of a population of cells can be instructive as to mechanism after chemical damage to DNA. If the insult is repairable, DNA from the nucleus should contain local breaks at sites where repair is initiated. As the efficient repair process proceeds, these breaks disappear and the integrity of the nuclear material is restored. In an SCGE assay under alkaline conditions of DNA treatment, this process would be observed by the initial presence of a long tail of electrophoretically mobile DNA fragments (analogous to a comet). As repair proceeds, the DNA is restored to progressively higher molecular weight material as evidenced by a shortened tail. Similarly, evidence of single or double strand breaks in DNA induced, for example, by ionizing radiation should become evident under neutral or alkaline treatment respectively. In the event that the agent produces crosslinks, nuclear DNA resists fragmentation and smaller tails will be observed on electrophoresis.

When Tice *et al.* (1997) examined mouse erythrocytes for intracellular distribution of DNA complement by the SCGE assay, they found no difference at any dose or over any period of exposure. There was no evidence that relates the percent of DNA that migrated from the nucleus in standard conditions (migrated DNA is the integrated intensity of fluorescence due to the presence of DNA in the tail divided by the integrated intensity of DNA in the total image. There is also no evidence of a dose-response relationship between tail length and length of *in vivo* exposure to phenolphthalein. An additional measure of the state of DNA in nuclei of phenolphthalein treated mice using the SCGE assay is tail moment, or the frequency of DNA in the tail multiplied by the tail length.

Although some significant differences were observed for various measures of DNA content (tail moment) in erythrocytes using the SCGE assay there was no consistent effect that could

be tied to a mechanism of genotoxic action. No clear relationship between the size of DNA in cells and the treatment the animals received could be established.

As pointed out above, the *p53* gene has been associated with the decision point for initiation of programmed cell death or proliferation. The nuclei of leukocytes or liver parenchymal cells undergoing early stages of apoptosis should show evidence of necrosis and pycnotic contraction, producing a very condensed distribution of DNA. The leukocytes examined in the SCGE assay failed to show this effect in an exposure dependent manner. At the end of the study period (24 weeks) Tice *et al.* (1997) examined the intracellular distribution of DNA in viable liver parenchymal cells and observed the inverse of what might be expected for the production of single strand breaks. Rather than evidence of DNA fragmentation, they observed an apparent increase of DNA integrity with dose of phenolphthalein up to 3,000 ppm. This result is suggestive of crosslinking of DNA, but there is no metabolic evidence that would support such a mechanism of action by phenolphthalein.

Finally, Tice *et al.* (1997) present evidence that examines the mode of micronucleus production by determining the presence or absence and frequency of kinetochore material in erythrocyte micronuclei. This test is designed to distinguish between micronuclei produced by clastogenic activity and micronuclei containing whole chromosomes which would be expected from an aneugen. Circulating PCEs and NCEs with large micronuclei could contain whole chromosomes. Evidence for the presence of whole or major segments of chromosomes can be shown by the presence of kinetochore proteins (Fuscoe *et al.*, 1996). In the event that a dose related increase in kinetochore staining material can be shown, a possible mechanism of action for phenolphthalein could be deduced. The results reported by Tice *et al.* (1997) suggest that in animals receiving the highest dose, a preponderance of micronuclei contained kinetochore material which might have resulted from numerical chromosomal damage (73%) with the remainder apparently related to chromosome breakage. This would tend to argue against phenolphthalein acting solely as a clastogen. The mitotic spindle poison cholchicine induces a high frequency of micronuclei which stain positive for kinetochore protein (Fuscoe *et al.*, 1996). In another study, however, Witt *et al.* (1995) clearly demonstrated that phenolphthalein can act as a clastogen in CHO cells treated *in vitro*.

The evidence of Tice *et al.* (1997) shows clear evidence of a treatment related increase in micronuclei in *p53*+/- female mice. However, there is only equivocal evidence for treatment

related DNA breakage or crosslinking. The absence of leukocytes with low DNA content does not suggest evidence of apoptosis in leukocytes or parenchymal liver cells related to treatment with phenolphthalein. The presence of greater than expected frequency of kinetochore staining material in micronuclei of one dose at one time point is only suggestive of aneugenic activity and would require significant additional research effort.

Dinnick *et al.* (1997) examined a number of tumors produced at different doses of phenolphthalein to determine whether there had been a loss of heterozygosity at *p53+/-* site in DNA form tumor cells. In 21 of 21 cases, Dinnick *et al.* (1997) reported evidence of loss or mutation of the wildtype allele. Harvey *et al.* (1993) have examined spontaneous mutants in *p53+/-* and compared frequency of loss or mutation of the functional allele in isolated tumors. The loss of heterozygosity was not associated with any spontaneously derived tumor type. This was determined by investigating the spontaneous tumors for the presence or absence of the remaining wildtype allele. Of 33 tumors that arose in heterozygous *p53+/-* mice, 18 (55%) exhibited clear loss of the wildtype allele. It is unlikely that the DNA from all lymphoma tumor cells would have lost their heterozygous genotype if toxicity or oxidative stress alone were responsible for the elevated tumor induction.

5.0 TUMORIGENESIS IN FEMALE *p53+/-* MICE

Table 4 shows the results for production of specific tumors or malignant neoplasms in either B6C3F1 or TSG *p53+/-*® mice treated with phenolphthalein at up to 12,000 ppm. The results of the NTP two year study (NTP 465) suggest that a specific spectrum of tumors should be found in *p53+/-* heterozygotes. As pointed out above, there have been several reports that support the hypothesis that *p53+/-* strains should demonstrate the same spectrum of tumors and that the relative time to tumor should be accelerated, providing the serum concentrations of phenolphthalein were similar in the two studies.

Table 4 Significant Malignant Neoplasms in B6C3F1 Mice in Either *p53*+/+ or TSG *p53*+/-® Animals

NTP 465 (2 Year)	DOSE ppm					
Sex/site	0	3000	6000	12000		
♂ Histocytic Sarcoma (all organs)	1/50	3/50	11/50 significant P=0.002	12/49 significant P= <0.001		
♀ Histocytic Sarcoma (all organs)	0/50	2/50	7/50 significant P=0.006	7/50 significant P=0.006		
♂ Malignant lymphoma (all organs)	6/50	8/50	12/50	8/49		
♀ Malignant lymphoma (all organs)	15/50	28/50 significant P=0.007	33/50 significant P<0.001	25/50 significant P=0.033		
♂ Malignant neoplasms (all organs)	19/50	29/50 significant P=0.036	35/50 significant P=0.001	29/49 significant P=0.028		
♀ Malignant neoplasms (all organs)	25/50	35/50 significant P=0.033	41/50 significant P<0.001	37/50 significant P=0.011		
♀ Ovary: Benign sex-cord tumor	0/50	7/49 significant P=0.006	6/50 significant P=0.013	5/50 significant P=0.028		
♀ TSG *p53*+/-	0	200	375	750	3000	12000
Malignant lymphoma	0	2/20	0/20	2/20	17/20	14/20
Total Primary neoplasms	3/20	3/20	1/20	2/20	18/20	16/20
Total malignant neoplasms	1/20	3/20	0/20	2/20	18/20	15/20

On this basis, the use of female *p53+/-* mice in feeding studies with phenolphthalein should lead to increased frequency of histocytic sarcomas, malignant lymphomas and benign ovarian sex-cord tumors. As general observation, it would be expected that an increase on malignant neoplasms should be observed in the exposed population.

The results of the feeding study with female *p53+/-* mice clearly show an increase in malignant lymphoma at the highest doses (3,000 and 12,000 ppm). Evidence presented in the reports shows that the serum concentration of phenolphthalein in animals examined at the termination of the study were within the ranges reported for the two year assay. The pathology evidence fails to show production of significant numbers of tumors at other sites. It is especially troublesome that the total malignant neoplasms detected would appear to show that the tumor type found was lymphoma. It would have been interesting to determine whether this tumor type which arises spontaneously in these heterozygous strains (32% to 57%) (Kemp *et al.*, 1994), would have been induced in male mice by phenolphthalein. The fact that no ovarian tumors or bone marrow tumors were reported suggests that *p53+/-* animals may not always respond with the tumor spectra of chemicals identified as carcinogenic in the two year assay.

6.0 CONCLUSIONS

It has been proposed that phenolphthalein produces tumors by a genotoxic mechanism, and that this could be due to the production of oxygen or hydroxyl radicals produced by futile metabolism. From the perspective of phenolphthalein acting as a genotoxic agent, this suggests specific consequences that may be analyzed through application of a variety of tests to examine the genetic material at several levels.

The most persuasive evidence of genotoxicity is that phenolphthalein has been repeatedly shown to produce micronuclei in polychromatic and normochromatic erythrocytes. There are a number of chemicals that can produce micronuclei but which are not considered rodent carcinogens (Shelby *et al.*, 1993). In the absence of clear evidence for a structural or metabolic mechanism of action for phenolphthalein, additional research should be initiated to clarify why micronuclei are produced.

Additional evidence of genotoxicity has been presented based on production of chromosomal

aberrations *in vitro* tests with Chinese hamster ovary (CHO) cells (Witt *et al.*, 1995) and a mixed population of Syrian hamster embryo (SHE) cells (Tsutsui *et al.*,1997). These assays are considered good evidence for direct genotoxic activity by a chemical. The fact that some form of mixed function oxidase activity is required to express this activity has led to the conclusion that some ultimate genotoxic intermediate of phenolphthalein is responsible for the production of chromosomal aberrations. The metabolism and disposition studies have shown no evidence of such an intermediate. One attempt to determine whether stable DNA adducts form with exposure to phenolphthalein in conditions that produce damage to chromosomes failed to produce any evidence of a stable DNA-phenolphthalein product. Disposition studies that show little labelled phenolphthalein remains in tissues after clearance, which suggests that additional searches for DNA adducts are unwarranted.

The contention of Tsutsui *et al.* (1997) that phenolphthalein can produce mutations in SHE cells is clearly not supported by the evidence presented.

The elegant work of Sipe *et al.* (1997) has provided a possible mechanism for the genotoxic effect of phenolphthalein through the production of oxygen radicals that could act directly on DNA to produce single strand breaks. The evidence of Tice *et al.* (1997) with the single cell gel electrophoresis (SCGE) has clearly not supported this mechanism. None of the leukocytes from any exposure regime revealed substantial DNA breaks at any time in the study. Rather the reverse may be argued, since DNA in later samples appeared to show evidence of very high molecular weight or possibly crosslinkages. There has never been any suggestion that phenolphthalein could act via mechanism that could lead to crosslinked DNA.

Other evidence, also provided by the SCGE assay with liver parenchymal cells, failed to show the presence of condensed nuclei. One of the initial steps of apoptosis is the production of pycnotic nuclei in affected cells. Some of the evidence from γ radiation of *p53+/-* and *p53-/-* cells was suggestive of induction of apoptosis in the nullizygous strains (Kemp *et al.*, 1994). It was proposed that if significant numbers of cells received DNA damage as a result of super oxide formation, apoptosis could be induced and these cells, programmed to die, would be removed from the population. Thus, if significant amounts of oxygen radicals were produced in response to phenolphthalein exposure, it was reasoned that cells showing evidence of apoptosis would be present. No effect of phenolphthalein was found in liver cells (liver is the site of most metabolism of phenolphthalein).

One of the interesting results of the molecular biological investigations was the finding that a greater number of the micronuclei produced in response to phenolphthalein treatment *in vivo* contained kinetochore proteins as shown by Tice *et al.* (1997) by fluorescent antibody staining. If the preponderance of nucleic acid material in the micromuclei were from fragmented chromosomes, it would be expected that few of these structures would be positive for kinetochore material. Tice *et al.* (1997) found just the opposite, suggesting that whole or large parts of chromosomes were in the micronuclei examined. This does not support the production of DNA breaks by clastogenic action, but may be more indicative of an effect of phenolphthalein on the mitotic spindle. It is recognized that *p53* protein has a function in delaying cell cycling into S phase by arresting cell progression at G1. It may be that additional investigation of the effects of phenolphthalein at cell division could provide more direct evidence of interference with spindle fiber assembly or action.

It is clear from the evidence of Dunnick *et al.* (1997) that tumor cells isolated from affected tissues of phenolphthalein treated mice have lost the wildtype allele from the *p53+/-* heterozygote. Evidence found by others who have examined similar lymphomas of spontaneous origin have found that these tumors arise in some cases without the loss of the wildtype allele from the chromosome. This results that there is some direct effect of phenolphthalein that increases the frequency of loss of the wildtype allele from targeted cells.

The evidence of tumor production in *p53+/-* female mice by exposure to high doses of phenolphthalein is strong yet troubling. There is ample evidence that chemicals that are known carcinogens accelerate the time to tumor in *p53+/-* mice when compared with wildtype B6C3F1 litter mates. Furthermore, the most persuasive evidence presented to date that argues for the suitability of this animal as a model for rodent carcinogenesis by genotoxic action is the similarity of tumor spectrum to that produced in the two year rodent study. With respect to phenolphthalein exposure, this is quite clearly not the case. The production of malignant lymphomas does match the evidence reported in the NTP study, but the tumors expected at other sites were not observed. While this does not bring the assay itself into question, there is a clear need to undertake additional study with other chemicals that are more toxic than genotoxic.

Appendix A

7.0 REFERENCES

Dunnick, J.K., Hardisty, J.F., Herbert, R.A., Seeley, J.C., Furedi-Machacek, E.M., Foley, J.F., Lacks, G.D., Stasiecwicz, S., and French, J.E. 1997. Penolthalein rapidly induces malignant hematopoietic tumors and loss of heterozygosity in the *p53* wildtype allele in heterozygous *p53* deficient (+/-) mice. Submitted for publication.

Donehower, L.A. 1996. Effects of *p53* mutation on tumor progression: recent insights from mouse tumor models. Biochimica et Biophysica Acta 1242:171-176.

Donehower, L.A., Harvey, M., Vogel, H., McArthur, M.J., Montgomery, C.A., Park, S.H., Thompson, T., Ford, R.J., and Bradley, A. 1995. Effects of genetic background on tumorigenesis in *p53*-deficient mice. Mol Carcin 14:16-22.

Fuscoe, J.C., Afshari, A.J., George, M.H., DeAngelo, A.B., Tice, R.R., Salman, T., and Allen, J.W. 1996. *In vivo* genotoxicity of dichloroacetic acid: evaluation with the mouse peripheral blood micronucleus assay and the single cell gel assay. Environ Molec Mutagen 27:1-9.

Griffin, R.J., Godfrey, V.B., and Burka, L.T. 1997. Metabolism and disposition of phenolthalein in male and female F344 and B6C3F1 mice. Submitted manuscript.

Harris, C.C. 1996. *p53* Tumor suppressor gene: from basic research laboratory to the clinic-an abridged historical perspective. Carcinogenesis 16:1187-1198.

Harvey, M., Vogel, H., Morris, D., Bradley, A., Bernstein, A., and Donehower, L.A. 1995. A mutant *p53* transgene accelerates tumour development in heterozygous but not nullizygous *p53*-deficient mice. Nature Genetics 9:305-311.

Harvey, M., McArthur, M.J., Montgomery, C.A., Butel, J.S., Bradley, A., and Donehower, L.A. 1993. Spontaneous and carcinogen-induced tumorigenesis in *p53* deficient mice. Nature Genetics 5:225-229.

Kemp, C.J., Weldon, T., and Balmain, A. 1994. *p53*-deficient mice are extremely susceptible to radiation-induced tumorigenesis. Nature Genetics 8:66-69.

Kerckaert, G.A., Brauninger, R., LeBoef, R.A., and Isfort, R.J. 1996. Use of the Syrian hamster embryo cell transformation assay for carcinogenicity prediction of chemicals currently being tested by the National Toxicology Program in rodent assays. Environ Health Perspect 104(5):1075-1084.

Lane, D.P. 1992. Cancer. *p53* guardian of the genome. Nature 358:15-16.

Mirsalis, J.C., Monforte, J.A., and Winegar, R.A. 1994. Transgenic animal models for measuring mutation *in vivo*. Crit Rev Toxicol 24(3):255-280.

Mirsalis, J.C., Monforte, J.A., and Winegar, R.A. 1995. Transgenic animal models for detection of *in vivo* mutations. Annu Rev Pharmacol Toxicol 35:145-164.

Parker, R.J., Hirom, P.C., and Millburn, P. 1980. Enterohepatic recycling of phenolthalein, morphine, lysergic acid diethylamide (LSD) and diphenylacetic acid in the rat: Hydrolysis of glucuronic acid conjugates in the gut lumen. Xenobiotica 10:689-703. Cited In: Sipe *et al.*, 1997.

Sands, A., Donehower, L.A., and Bradley, A. 1994. Gene-targeting and the *p53* tumor-suppressing gene. Mutat Res 307:557-572.

Shelby, M.D., Erexson, G.L., Hook, G.J., and Tice, R.R. 1993. Evaluation of a three-exposure mouse bone marrow micronucleus protocol: results with 49 chemicals. Environ Mol Mutagen 21:160-179.

Sipe, H.J., Corbett, J.T., and Mason, R.P. 1997. *In vitro* free radical metabolism of phenolthalein by peroxidases. Metabolism and Disposition 25:468-480

Tennant, R.W. 1994. Introduction. Mutat Res 307:427-428.

Tennant, R., W., Rao, G.N., Russfield, A., Seilkop, S., and Braum, A.G. 1993. Chemical effects in transgenic mice bearing oncogenes expressed in mammary tissue. Carcinogenesis 14(1):29-35.

Tennant, R.W., Hansen, L., and Spalding, K. 1993. Gene manipulation and genetic toxicology. Mutagenesis 9(3):171-174.

Tennant, R.W., French, J.E., and Spalding, J.W. 1995. Identifying chemical carcinogens and assessing potential risk in short-term bioassays using transgenic mouse models. Environ Health Perspect 103(10):942-950.

Tice, R.R., Fruedi-Machacek, M., Satterfield, D., Udumudi, A., Vasquez, M., and Dunnick, J. 1997. Genotoxicity of chronically ingested phenolthalein in female heterozygous TSG-*p53*® mice. Submitted manuscript.

Tsutsui, T., Tamura, Y., Yagi, E., Hasegawa, K., Tanaka, Y., Someya, T., Hamaguchi, F., Yamamoto, H., and Barrett, J.C 1997. Cell transforming activity and genotoxicity of phenolthalein in cultured Syrian hamster embryo cells. Submitted manuscript.

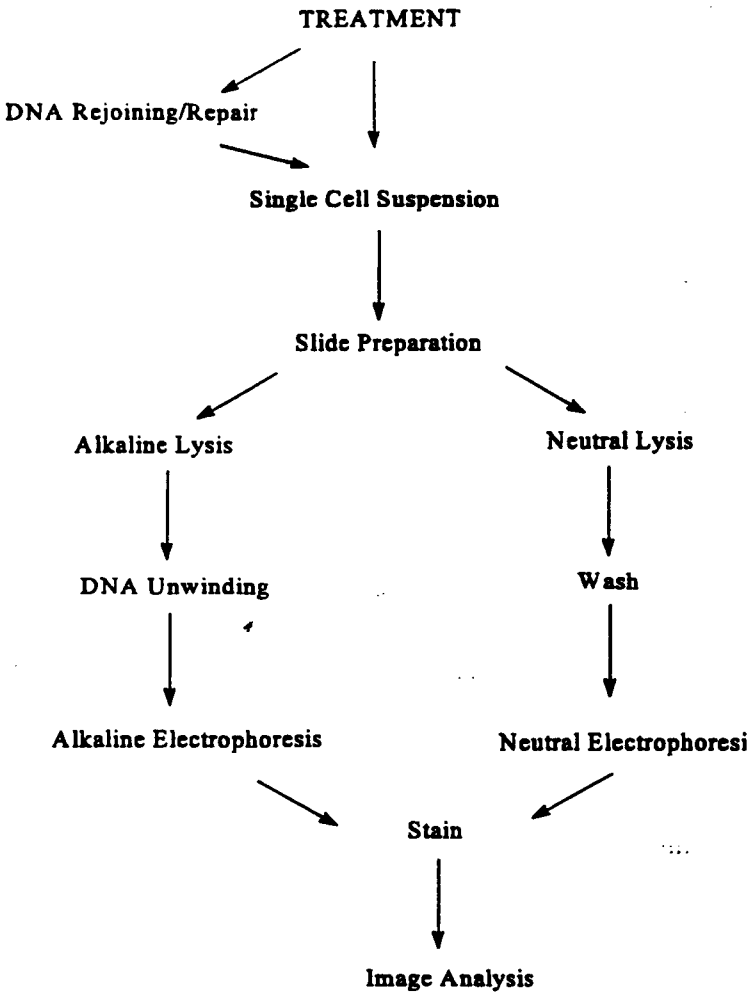
Tsutsui, T., Maizumi, H., and Barrett, J.C. 1984. Amitrole-induced cell transformation and gene mutations in Syrian hamster embryo cells in culture. Mutat Res 140:205-207.

Witt, K.L., Gulati, D.K., Kaur, P., and Shelby, M.D. 1995. Phenolthalein: induction of micronucleated erythrocytes in mice. Mutat Res 341:151-160.

APPENDIX B:

THE SCGE ASSAY

SCGE



- Comets form as ends of negatively charged DNA are freed to migrate towards the anode in an electric field.

- A high pH (> 12.3) is utilized to facilitate denaturation, unwinding and expression of single strand breaks that become apparent only after exposure to alkali (alkali labile lesions).
- Neutral electrophoresis conditions show evidence of double strand breaks.

SCGE methodology:

- Cells to be evaluated must be distinctly separated - usually a suspension of individual cells; trypsinization and scraping usually induce DNA damage; incubation of lymphocytes for 1h in PBS results in damage.

- Accurate measurement of induced damage requires that samples be isolated and processed without additional repair or permitting the production of additional breaks

- Cell cycle status may be important: Complications may occur because of S-phase DNA. Replicating structures will appear as breaks in the **alkaline assay**. In the **neutral assay,** sites of replication increase tangling and reduce DNA migration.

- Cells are suspended in low melting point agar (0.5 to 1%). Cast gels are of two types: (1) single layer, and (2) sandwich. Sandwich gels consist of three distinct layers.

- Greatest variability of the assay relates to electrophoresis conditions

- In cases where DNA **damage is minimal** and where very low doses are used to induce damage, a closer relationship between tail length and dose exists

- Accurate assessment of damage depends on:
  1. lysis conditions
  2. pre-electrophoresis wash
  3. residual salt which can inhibit DNA migration...removal of solutions used in the lysis step is important. This has an effect on the voltage required to carry out electrophoresis.
  4. recirculation of buffer during electrophoresis or prerunning gel to remove any contaminant salt.

- The comet assay can determine whether an agent produces DNA strand breaks, but it can not measure the fidelity of repair of those breaks.

- Radiation damage or the occurrence of endogenous oxidative damage has been widely examined using the comet assay.

- Apoptotic cells: The extent of DNA damage in apoptotic cells is so great that they are easily distinguished visually from undamaged cells.

- **Tail moment**: The product of the percentage of DNA in the tail **and** the length of the tail are used to produce a quantitative parameter of DNA breakage.

- Requirements: Cell # : $<2 \times 10^5$; assay performed in a few hours; similar sensitivity to alkaline elution assay but considerably shorter time frame; equipment not more exotic than for other short-term tests.

Opinion on the safety of phenolphthalein as an ingredient of OTC laxative preparations

Author : Dr. F.J.C. Roe

Date : 28th August 1996

1. Introduction

1.1 The purpose of this opinion is to update the Medicines Control Agency (MCA) with respect to deliberation in the USA concerning the safety of phenolphthalein and in particular to comment on the possible value of the p53 transgenic mouse test which is presently in progress in the USA.

1.2 The preliminary publication in the USA in December 1995 of the findings in carcinogenicity tests involving the oral exposure of F344/N rats and B6C3F$_1$ mice to phenolphthalein (NTP, 1995) raised concern with regard to the safety of over-the-counter (OTC) laxative preparations which contain phenolphthalein as an active ingredient.

1.3 Inter alia, NPT concluded that the findings in the two-year feeding studies provided clear evidence of carcinogenic activity in male rats (adrenal medullary tumours and renal tumours and clear evidence of carcinogenicity in both male and female mice (histiocytic sarcoma and malignant lymphoma)

1.4 In the UK, the Medicines Control Agency (MCA) has requested further information about the new US studies when these are available.

1.5 On April 2nd 1996, 14 members of the Carcinogenicity Assessment Committee in the USA met with representatives of the FDA, Industry and NTP to consider the potential cancer risk for humans from the consumption of phenolphthalein-containing laxative preparations. Minutes of that meeting at which no unanimous conclusions were reached are attached as ANNEX C.

1.6 In the present opinion, the relevance of the findings in the NTP rodent tests to the safety-in-use for humans of OTC laxative preparations containing phenolphthalein is evaluated and discussed.

1.7 My competence to provide this opinion is supported by my curriculum vitae (ANNEX A)

2. The results of the deliberations of the Carcinogenicity Assessment Committee

2.1 At the meeting on 2nd April 1996 of the Carcinogenicity Assessment Committee, answers were sought to 5 questions:-

(1) Do the carcinogenicity studies as conducted provide a valid assessment of the carcinogenic potential of phenolphthalein?

(2) Do the studies provide evidence of trans-species tumorigenic potential for phenolphthalein?

(3) Do the studies addressing genotoxic potential and comparative metabolism and exposure provide information of potential relevance to human risk?

(a) Do you conclude phenolphthalein is a likely genotoxin?

(b) Is the metabolism of phenolphthalein and systemic exposure to phenolphthalein under the conditions of the bioassay in rodents sufficiently similar to that in humans under the conditions of standard use?

(4) Do you conclude that, based on the tumour findings in the bioassay, the genotoxicity results and comparative metabolism and exposure information, that the studies conducted provide evidence for carcinogenic potential of phenolphthalein relevant to human use?

(5) Although the OTC division will make a decision based on the information currently available are there any specific studies which you believe should be conducted to further clarify this issue?

2.2 Complete consensus was not reached in relation to the answers to any of these questions, the voting being as indicated in the following sections:-

2.3 Answer to Question 1: 12 "Yes" and 1 "No"
with several members who voted "Yes" believing that the dose levels studied were excessive (i.e. in excess of the MTD)

2.4 Answer to Question 2:
For the rat - 5 "Yes" and 1 "No"
For the mouse - 9 "Yes" and 4 "No"
More members were concerned about the thymic lymphoma (9 members) and the histiocytic sarcomas (7 members) in mice than about the renal adenomas (4 members) and phaeochromocytomas (1 member) in rats.

General renal toxicity in rats was considered to have predisposed to the occurrence of renal tumours in rats and stress due to high dose was thought to have predisposed to adrenal phaeochromocytomas in rats.

2.5 Answer to Question 3: 8 "Yes" and 3 "No"

2.5.1 Answer to Question 3a: 8 "Yes" and 3 "No"
However, 4 of those who voted "Yes" thought that the positive results in tests for genotoxicity might be artefacts due to high dosage or unusual study design, and that better data would be useful.

The 3 members who voted "No" pointed out that the evidence of genotoxicity related solely to clastogenic activity and that this could have been secondary to toxicity.

Six members considered that further studies using lower doses were needed before one could assess the genotoxic potential of phenolphthalein for humans.

2.5.2 Answer to Question 3b: 7 "Yes" and 6 "No"
Those who voted "No" considered that the level of systemic exposure in rodents was excessive compared with that in man. For this reason the results of the rodent studies cannot be extrapolated confidently to conditions of low exposure of humans.

2.6 Answer to Question 4: 5 "Yes" and 7 "No"

Those voting "Yes" considered there to be a real carcinogenic potential of phenolphthalein for humans if the chemical really is genotoxic. However, they considered that further tests for genotoxicity are needed.

Those voting "No" considered it unlikely that phenolphthalein is genotoxic or that it is carcinogenic for humans, except, possibly, under conditions of chronic misuse (e.g. excessive daily doses over long periods)

2.7 Answer to Question 5: 9 "Yes" and 4 "No"

The additional studies suggested were:-

(i) Human epidemiologic study

(ii) Assessment of saturation of metabolism/detoxification in relation to genotoxicity

(iii) More complete exposure data and metabolism data under the same conditions as the carcinogenicity studies

(iv) A mouse lymphoma study for point mutations

(v) A human cell transformation study

(vi) A study to see if DNA adducts are formed in response to exposure to phenolphthalein over a range of doses

(vii) A p53 transgenic mouse study.

3. The present authors opinion of the results of the NTP rodent feeding studies

3.1 Early in February 1996 I prepared a detailed opinion of the findings in the NTP studies. A copy of this opinion is attached as ANNEX B.

Inter alia, I pointed to the evidence supporting the view that the higher incidence of adrenal medullary tumours in rats exposed to high doses of phenolphthalein might well have been secondary to a disturbance of calcium homeostasis (see paragraphs 18.6.1 to 18.6.8 of Annex B). In other words, a well-known non-genotoxic carcinogenic mechanism could account for the enhancement of the incidence of the adrenal medullary tumours in rats.

3.2 As far as the increased incidence of renal tumours in male rats is concerned, the possibility that this was secondary to increased cell turnover in renal tubule cells because of a treatment-related effect on the incidence/severity of chronic progressive nephropathy is hypothesised by NTP (1995). The same hypothesis was seemingly accepted by 9 of the 13 members of the Carcinogenicity Assessment Committee at its meeting on 2nd April 1996 (see ANNEX C, page 8)

3.3 It is well known that oestrogens increase the incidence of malignant lymphoma, histiocytic sarcoma and many of the non-neoplastic lesions seen in the 2-year NTP mouse study. In my opinion (ANNEX B) I express the conclusion that the effects of exposure of mice to phenolphthalein which NTP concluded constituted "clear evidence of carcinogenicity" (namely, increased incidence of histiocytic sarcoma and malignant lymphoma) along with effects on many other non-neoplastic changes can be attributed to the known oestrogen agonist and/or antagonist activities of phenolphthalein brought about by its interaction with oestrogen receptor protein. Thus, as in rats, the adverse effects of high (toxic) doses of phenolphthalein on tumour incidence in mice are explained in terms of known non-genotoxic mechanisms of carcinogenesis.

3.4 Additional references to the effects of oestrogens in increasing the risk of development of leukaemia in mice are Kirschbaum (1956), Gardner et al (1944), Gardner et al (1940), Higgins et al (1950) and Murphy (1944). In considering these papers it needs to be realised that in the 1940's and 1950's histiocytic sarcomas would have been included in the overall category of "lymphoreticular neoplasms" of "malignant lymphoma".

3.5 A further conclusion implied by my opinion of the NTP data (ANNEX B) is that studies of the effects of realistic dose levels of orally administered phenolphthalein to rats and mice would not lead to enhanced incidence of any kind of neoplasm because they would not cause the disturbances of homeostasis that predispose, by non-genotoxic mechanisms, to the types of tumours seen in excess in the NTP studies.

4. Opinion on what further research needs to be done

4.1 Seven suggestions for further studies were discussed by the Carcinogenicity Assessment Committee (see paragraph 2.7 above). However, of these the only one that has so far been followed up appears to be the last, namely, a p53 transgenic mouse study.

4.2 Personally, I do not understand the rationale behind the suggestion that a p53 knockout transgenic mouse study (vii) should be conducted. My reasons are as follows:-

4.2.1 According to the two-stage model of carcinogenesis, the first, tumour-initiating, stage involves genotoxic damage. Wild type p53 protects against cancer development by promoting the premature senescence of mutant cells. The protection is hampered in mice that are heterozygotic for wild type p53 gene loss and protection is abolished in p53 null mice. P53 knockout transgenic mice are prone to the spontaneous development early in life of a variety of neoplasms and mice that are heterozygotic for p53 loss are intermediate between mice that are homozygous for p53 loss and normal mice in respect of if and when they spontaneously develop tumours (Harvey et al 1993)

4.2.2 The second, tumour-promoting, stage of carcinogenesis involves enhancement of clonal expansion of mutant cells.

4.2.3 Tumour progression is the term applied to the process whereby tumours that are initially benign become more and more malignant in their behaviour. This phenomenon could be brought about by successive mutations, and particularly by a mutation of, or loss of, the wild type p53 gene.

4.2.4 Kemp et al (1993) using the two-stage mouse skin carcinogenesis model reported that p53 knockout had no effect on response to tumour-initiating chemicals and no effect on response to tumour-promoting chemicals. Its effect is confined to enhancing tumour progression.

4.2.5 A weakness with the studies reported by Kemp et al (1993) however, is that the investigators assumed that malignant skin tumours in mice usually arise by progression from benign skin tumours. In my personal rather extensive experience of two-stage mouse skin carcinogenesis, however, the truth is that most malignant tumours appear to arise de novo and not in pre-existing benign papillomas - certainly not in benign tumours that are big enough to be seen by the naked eye or felt by palpation.

4.2.6 In the light of these considerations I do not see how a study involving the exposure of p53 knockout transgenic mice can throw any critical light on whether phenolphthalein poses a cancer risk either for normal animals or for humans. If such a study were conducted in homozygous p53 knockout transgenic mice, the early development of tumours in high incidence in untreated controls would obscure any enhancing effect of phenolphthalein on tumour development. Furthermore, if phenolphthalein enhanced the development of tumours in mice that were heterozygous for p53 gene loss, the effect would according to Kemp et al (1993) be attributable to acceleration of tumour-progression and not to either increased mutation or tumour promotion.

4.2.7 Thus, whatever the results of the proposed study in p53 transgenic mice, I cannot see how they can throw any useful light on possible cancer risk for man.

4.2.8 A further point is that whatever the results of such a study, there exists no basis for regarding the test system as having been validated for the purposes of distinguishing between chemicals that pose a carcinogenic risk and chemicals that pose no such risk. It may be the case, for instance, that carcinogenically innocent substances such as sugar and salt enhance tumour development in p53 knockout transgenic mice. At present we simply do not know whether this is true or not.

4.2.9 I suspect that the thinking behind the recommendation that a p53 transgenic mouse study be carried out, stems from the test being quicker and cheaper than

further 2-year rodent studies involving realistic exposure to phenolphthalein. I do not regard this recommendation as scientifically sound.

4.2.10 From correspondence that I have had with Dr. Gary Boorman (National Institute of Environmental Health Sciences, USA; and Head of the National Toxicology Program) in which he drew my attention to papers by Tennant et al (1995) and Hansen et al (1995), I deduce that the strategy of NIEHS at present is to conduct 2-year bioassay studies and 30-week studies using transgenic mice in parallel. I also deduce that NIEHS does not believe that there is presently enough knowledge for reliance to be put solely on the use of transgenic mice.

5. Summary of present opinion

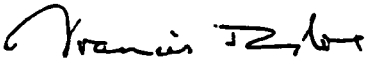
5.1 The available data for tests of phenolphthalein for genotoxicity are inadequate for indicting the compound as genotoxic. Positive results for clastogenicity have been reported in in vitro tests involving exposure to cytotoxic concentrations of phenolphthalein and in an in vivo micronucleus test of non-standard design. Conventional in vitro tests for gene mutation have given negative results as has a test for Sister Chromatid Exchanges (see Annex B, section 14). The undertaking of a further in vivo mouse micronucleus test of conventional design should be considered.

5.2 In section 4.2 I list reasons why I do not regard the p53 knockout transgenic mouse test as having been validated for the purpose of identifying potential human carcinogens.

5.3 In section 3 I summarize the reasons set out in my earlier opinion (see ANNEX B) for rejecting the conclusion of NTP (1995) that the findings in the 2-year F344/N rats and B6C3F$_1$ mice carcinogenicity studies provide clear evidence of carcinogenicity at any site. The fundamental error in the design of these studies was to confine attention to the responses of animals to dose levels close to the maximum tolerated dose (MTD) which is vastly in excess of the dose levels

received by humans consuming laxative preparations containing phenolphthalein.

5.4 There is presently no non-controversial evidence either of true genotoxicity or of animal carcinogenicity relevant to man. This being so, it is my considered opinion that there is no justifiable basis for curtailing the incorporation of phenolphthalein in OTC laxative preparations.

Signed: 

Francis J.C. Roe

DM, DSc, FRCPath, FATS

Date: 28th August 1996

Annexes

ANNEX A	Curriculum vitae of Dr. Francis J.C. Roe
ANNEX B	Dr. F.J.C. Roe's opinion on the findings in laboratory tests of phenolphthalein for toxicity and carcinogenicity (dated 5th February, 1996)
ANNEX C	Minutes of meeting of Carcinogenicity Assessment Committee

ANNEX A

CURRICULUM VITAE

FRANCIS J.C. ROE

Name:	Francis John Caldwell Roe	
Date of birth:	16th August 1924	
Marital status:	Married (Brenda Joan) with 4 children	
Education:	1933-1943	St. Olave's Grammar School, London
	1943-1948	Wadham College, Oxford and The London Hospital Medical College
Degrees and qualifications:	B.A. (Oxon)	1945
	B.M., B.Ch. (Oxon)	1948
	M.A. (Oxon)	1950
	D.M. (Oxon)	1957
	D.Sc. (London)	1965
	F.R.C. Path.	1967
	F.A.T.S.	1990
Scholarships:	1945	Price University Scholarship in Anatomy and Physiology, London Hospital Medical School.
	1945	Open Scholarship, St. George's Hospital, London (not taken up)
	1945	Lord Kitchener Scholarship
Award:	Gold Medal presented by the Centro Sociale Studio Precancerosi of Rome in recognition of his research in the field of carcinogenesis (1968)	
Prizes:	1979	The Royal Society of Medicine's Ver Heyden de Lancey Prize for Medicine and Art
	1980	Ver Heyden de Lancey Medical Art Society Prize for Sculpture
Lectures:	Leon Golberg Memorial Lecture at Royal College of Physicians, London, February 3rd 1993	
	Chappel Memorial Lecture, Guelph, Ontario, Canada, April 7th 1993	

Appointments:	1948-1949	House Appointment at the London Hospital
	1949-1951	Trainee Pathologist, then Graded Pathologist RAMC
	1951-1961	Lecturer then Senior Lecturer in Department of Cancer Research, London Hospital Medical College. 1956-1957 British-American Exchange Fellow in Cancer Research awarded by the British Empire Cancer Campaign for Research. Year spent at McArdle Memorial Laboratories, Madison, Wisconsin.
	1961-1971	Reader in Experimental Pathology (University of London) at Chester Beatty Research Institute Also Associate Pathologist (Hon. Consultant) to the Royal Marsden Hospital.
	1971-1973	Research co-ordinator, Tobacco Research Council, London.
	1973-April 1992	Independent Consultant in Toxicology, Experimental Pathology and Cancer Research.
	From Nov 1 1992	Partner in Roe Partners Pathtox Services
Experience of General Practice in Medicine:	1951-1966	Considerable experience in general medical practice as part-time locum.
Teaching Experience:	Supervision of Ph.D. Students. 10-20 lectures per year to audiences of widely different types.	
Research interests:	General toxicity and potential carcinogenicity of foods, food additives, food contaminants, drugs, tobacco smoke, air, water and soil pollutants and industrial chemicals. Pathology of laboratory animals Mechanisms of carcinogenesis Epidemiology of cancer Cancer prevention	

Membership of Committees:

Member of World Health Organisation Expert Advisory Panel on Food Additives (until 1988)

British Association for Cancer Research (until 1965)

Committee on Carcinogenicity, Department of Health and Social Security (until March 1989)

Committee on Toxicity, Department of Health and Social Security (until March 1989)

British Toxicological Society (since 1982)

Nuffield Foundation: Food Safety Committee (1962-1970)

Member of Council and member of the Executive Establishments and Cancer Research Committees of the Marie Curie Foundation. Also member of the Board of the Institute of Oncology of the Marie Curie Foundation.

Board of Management of the British Occupational Hygiene Society (until 1972)

Medical Advisory Committee of Women's National Cancer Control Campaign (since 1969)

WHO Scientific Group on principles for the testing and evaluation of drugs for carcinogenicity (December 1968)

IARC Working Group Evaluation of the Carcinogenic Risk of Chemicals to Man: Inorganic and Organometallic Compounds (Chairman - Lyon, November, 1972)

Laboratory Animal Science Association (LASA) (until 1968)

International Committee on Laboratory Animals (until 1968)

Experimental Pathology Club (from 1969)

Medical Art Society (Member since 1969, President 1975-1980)

Membership of Editorial Boards:	British Journal of Cancer (until 1983)
	International Journal of Cancer (until 1968)
	Food and Chemical Toxicology (formerly Food and Cosmetics Toxicology) (since 1963) Currently "Review Editor"
	Laboratory Animals (until 1971)
	Cancer Section: Excerpta Medica (until 1981)
	Human and Experimental Toxicology (1985-1991)
	Experimental and Toxicologic Pathology (from January 1992)
	Indoor Environment
	Human and Ecological Risk Assessment (1994)
Membership of Societies:	Royal Society of Medicine
	British Association for Cancer Research
	British Medical Association
	Royal College of Pathologists (Fellow)
	Society of Toxicology
	European Society of Toxicology
	Experimental Pathology Club
	Laboratory Animal Science Association
	Marie Curie Foundation
	Research Defence Society
	European Association for Cancer Research
	British Occupational Hygiene Society
	Medical Art Society
	Academy of Toxicological Sciences (Fellow)
Other interests:	Portrait Sculpture (Exhibited at Society of Portrait Sculptors 1967 and 1968 and at Medical Art Society regularly since 1967).
Club:	Athenaeum

Books published:	1966	The Biology of Cancer (with E.J. Ambrose) L. Van Nostrand Co. Ltd. (London) 237 pages NB Second edition - 1976
	1967	The Prevention of Cancer (with R.W. Raven) Butterworths (London) 397 pages
	1969	Pathology of Laboratory Rats and Mice (with E. Cotchin) Blackwell Scientific Publiations (Oxford) Approx. 850 pages
	1970	Metabolic Aspects of Food Safety Blackwell Scientific Publications (Oxford) 612 pages.
	1977	Metronidazole. Proceedings of the International Metronidazole Conference, Montreal, Quebec, Canada, May, 1976
	1983	Microbiological Standardization of Laboratory Animals Ellis Horwood
	1985	The Chemical Industry and Health of the Community. Royal Society of Medicine International Congress and Symposium Series No. 82.
	1989	Assessment of Inhalation Hazards: Integration and Extrapolation Using Diverse Data. ILSI Monographs. Springer-Verlag. 382pp

Other published work: 820 signed or unsigned articles, book reviews etc. in medico-scientific journals (78-page list available on request)

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Dr. C. J. Kemp,
Cancer Research Campaign Beatson Laboratories,
Garscube Estate,
Switchback Road,
Bearsden,
Glasgow G61 1BD

10th June, 1996

Dear Dr. Kemp,

I am writing to request your comments on the interpretation of studies based on the use of p53 knockout (transgenic) mice for the evaluation of chemicals for carcinogenicity.

From your paper in Cell (74:813-822, 1993) I understood that p53 knockout does not increase the sensitivity of mice to the tumour-initiating effect of mutagens, nor does it increase sensitivity to tumour promoters of skin carcinogenesis in mice. On the contrary, it enhances progression from benign to malignant.

My experience with the two-stage mouse skin carcinogenesis model during the 1950s and 1960s led me to conclude that most squamous carcinomas that arise relatively late in such studies do so de novo and very few arise in benign papillomas of sizes visible to the naked eye. Do you really have strong evidence that p53 knockout enhances progression from visible papilloma to squamous carcinoma?

Apart from this, I have what I think is a more important question. In the USA it seems that p53 knockout mice are presently being used as a substitute for 2-year rat and mouse studies for the assessment of chemicals for carcinogenic potential. My question is, what does a positive result in such a study mean in terms of possible cancer risk to humans from exposure to a test substance - particularly if the substance in question is not genotoxic according to a conventional array of tests for gene mutation and

Phenolphthalein : Opinion on the findings in laboratory tests for toxicity and carcinogenicity

Author : Dr. F. J. C. Roe, DM, DSc, FRCPath, FATS
Date : 8th February, 1996

1. Introduction to the present opinion

1.1 My comments have been requested on the findings in carcinogenicity tests on orally administered phenolphthalein (PH) carried out in the USA (as part of the National Toxicology Program : NTP TR 465) in F344/N rats and $B6C3F_1$ mice. The findings in these tests have so far only been published in draft form. The scheduled Peer Review date was December 5-6, 1995. I do not presently know the outcome of this peer review.

1.2 Phenolphthalein is an ingredient in many different over-the-counter laxative preparations and it was for this reason that it was selected for study under the NTP.

1.3 The studies carried out and reported on were:-

(i) Tests for genotoxicity on Salmonella typhimurium, cultured Chinese hamster ovary cells, and mouse peripheral blood for micronuclei;

(ii) Feeding tests of 14 days, 13 weeks and 2 years duration in F344/N rats;

(iii) Feeding tests of 14 days, 13 weeks and 2 years duration in $B6C3F_1$ mice.

No change

1.4 In the Introduction to the draft NTP report, successive sections concern:-

(i) the chemical and physical properties of phenolphthalein (PH) (see Section 2);

(ii) the production and uses of PH (see Section 3);

(iii) occupational exposure to PH (see Section 4);

(iv) the regulatory status of PH in the USA (see Section 5);

(v) the pharmacokinetics, absorption, distribution metabolism and excretion of PH (see Section 6);

(vi) the pharmacology of PH (see Section 7);

(vii) the general toxicity of PH in experimental animals (see Section 8);

(viii) the general toxicity of PH in humans (see Section 9);

(ix) the findings in laboratory tests for reproductive and developmental toxicity and observations in humans relevant to such manifestations of toxicity (see Section 10);

(x) the findings in earlier animal tests relevant to the possible carcinogenicity of PH (see Section 11);

(xi) the findings in human epidemiological studies relevant to carcinogenicity (see Section 12);

and (xii) the findings in previously reported tests of PH for genotoxicity (see Section 13).

1.5 The Introduction to the draft NTP report is followed by a Materials and Methods Section which is relevant to all the in vivo rat and mouse studies. Thereafter follows the Results Section which discusses, successively, the findings in the 3 rat studies and those in the 3 mouse studies. Finally comes a 16-page Discussion & Conclusions Section, a 13-page list of References and 15 Appendices : A-L.

1.6 In the present opinion I first summarise and discuss the contents of topics (i) - (xii) listed above in paragraph 1.4. Secondly, I consider the findings in the tests for genotoxicity (see item (i) in paragraph 1.3). Thirdly, I summarise and consider in some detail the findings in the six in vivo studies which constitute the main part of the NTP Report (see items (ii) and (iii) in paragraph 1.3). Finally, in the light of all these considerations, I express my own opinion with regard to the possible carcinogenic risk to humans posed by the incorporation of phenolphthalein in laxative formulations.

1.7 My competence to express an opinion on this topic is supported by my curriculum vitae which is attached as ANNEX A.

2. The chemical and physical properties of PH [see paragraph 1.4 (i)]

These provide no grounds for suspecting that PH is a genotoxin or a carcinogen other than, perhaps, the presence of a lactone ring in its structure.

3. The production and uses of PH (see paragraph 1.4 (ii)]

In the USA approximately 250 tons of PH are produced each year. It is not clear how much of this is produced for inclusion in laxative tablets - but presumably only a small percentage of the total. Daily doses for children aged 6-11 range from 30 to 60 mg and for adults from 30 to 270 mg. The use of stimulant laxatives such as PH is not recommended for children aged less than 6 years. Like other stimulant laxatives (i.e. laxative cathartics) PH is habit-forming and chronic usage can lead to dependence.

4. Occupational exposure to PH [see paragraph 1.4 (iii)]

In the USA, it has been calculated that 75,000 people are occupationally exposed to PH.

5. Regulatory status of PH in the USA (see paragraph 1.4 (iv)]

In the USA the Food & Drug Administration has hitherto categorized PH as GRAS (Generally Recognized As Safe - see Federal Register 1975).

6. Pharmacokinetics, absorption, distribution & excretion of PH [see paragraph 1.4 (v)]

Studies in various species of animal indicate that PH is readily absorbed from the intestine; extensively conjugated with glucuronic acid; excreted in the bile, urine, faeces and milk; distributed via the blood and lymph throughout the body (N.B. It is not stated whether it passes the blood-brain barrier); and

subject to enterohepatic recirculation after excretion in the bile. Glucuronide conjugation activity is stimulated by phenobarbital in adult rats. In dogs, it has been reported that 50% of the label was recovered in the faeces and 36% in the urine after an oral dose of 4.8 mg [^{14}C]-PH/kg bw (uniformly labelled) within 72 hours. No [^{14}C]carbon dioxide was recovered after mice were exposed to PH labelled on the non-aromatic carbon of the lactone ring, and 96% of the radioactivity was recovered in the urine and faeces within 48 hours. Thirty minutes after dosing, the highest levels of radioactivity were found in the liver. It has been reported that the label of [^{14}C]-labelled PH crosses the plactental barrier in mice. However, transplacental passage appears to be very limited in dogs.

In humans, there is apparently only limited absorption from the gut and most of the absorbed PH is excreted in the urine as phenolic-hydroxyglucuroride or sulphate conjugates. Some conjugated PH is excreted in the bile and enterohepatic recirculation probably contributes to the laxative effect of PH, which is not seen in patients with obstructive jaundice or in animals after ligation of the common bile duct.

In my opinion, none of the available data concerning the pharmacokinetics, absorption, distribution or excretion of PH constitute grounds for suspicion of possible carcinogenic risk.

7. <u>The pharmacology of PH</u> [see paragraph 1.4 (vi)]

PH stimulates the secretion of bile and increases the

fluid content of the intestine via an affect on the sodium pump resulting in an inhibition of water absorption. Also, PH stimulates prostaglandin biosynthesis in the colon of rats and probably also of humans.

The only information on the pharmacology of PH that gives rise grounds for suspecting it of posing a carcinogenic risk is its possession of weak oestrogenic activity (see paragraph 10)

8. The general toxicity of PH in experimental animals (see paragraph 1.4 (vii)]

The oral administration of up to 50 mg PH/kg bw/day for 135 days to female mice led to no evidence of toxicity or of histopathological changes in the liver, kidneys or gastrointestinal tract. These findings, along with observations on dogs exposed to PH by the intravenous route, rats exposed to PH by intraperitoneal injection, on cultured liver cells provide no grounds for suspecting it of posing a carcinogenic risk.

9. The general toxicity of PH in humans [see paragraph 1.4 (viii)]

Apart from gastrointestinal symptoms and faintness, toxicity is not a problem in humans taking PH except sometimes in subjects who abuse its use. Hypersensitivitiy has been reported in specially susceptible or atopic individuals. A few cases of fatal PH poisoning of children have been reported, the cause of death being excessive diarrhoea and dehydration etc.

None of the data relating to the toxicity of PH for humans as cited in the NTP draft report are in any way suggestive that the use of PH poses a risk of carcinogenesis.

10. Reproductive and development toxicity [see paragraph 1.4 (ix)]

A 3-generation study in mice reported in 1965 gave negative results in respect of teratogenicity and adverse effects on reproductive parameters. However, studies in immature rats have pointed to PH having weak oestrogenic activity (Bitman & Cecil, 1970; Nieto et al, 1990).

It is probable that this oestrogenic activity is relevant to some of the effects observed in mice in the 2-year feeding study. [See Section 19]

11. The findings in earlier animal tests relevant to the possible carcinogenicity of PH [see Section 1.4 (x)]

No subcutaneous tumours arose in mice given 13-once-weekly subcutaneous injections of phenolphthalein phosphate (dose unspecified) and thereafter observed without treatment for 8 months (Haddow and Horning, 1960).

Prior to the NTP study there have been no studies of modern design involving exposure to phenolphthalein by the oral route.

12. The findings in human epidemiological studies relevant to carcinogenicity [see paragraph 1.4 (xi)]

Kune (1993), in a study of 1408 subjects, saw no significant increased risk of colorectal cancer in PH laxative users. Earlier, Wu et al (1987) reported finding the risk of colorectal cancer to be no higher among laxative users than laxative non-users in a retirement community of nearly 12,000 residents.

13. The findings in previously reported tests of PH for genotoxicity [see paragraph 1.4 (xii)]

Negative findings have been reported for the induction of DNA damage in repair-deficient strains of Bacillus subtilis, and in tests for mutagenicity in Salmonella typhimurium in two different laboratories. However, Dietz et al (1992) reported finding significantly increased numbers of micronucleated erythrocytes at the end of a 13-week toxicity test of PH in mice. Follow-up tests confirmed that oral exposure of $B6C3F_1$ mice to a least 2000 mg/kg bw/day PH for at least 2 days led to evidence of this manifestation of chromosomal damage (Witt et al, 1995). The same investigators also reported (i) increased micronuclei in Swiss mice exposed for 14 weeks to 120 mg/kg bw/day PH and (ii) an increased incidence of chromosomal aberrations in Chinese hamster ovary (CHO) cells in vitro.

14. Details of tests for genotoxicity [see Annex E of Draft Report]

14.1 NTP ANNEX E provides details of the S.typhimurium tests reported by Mortelmans et al (1986). The protocols are satisfactory and results negative.

14.2 The in vitro studies in CHO cells for sister chromatid exchanges and chromosomal aberrations reported by Galloway et al (1987) and Witt et al (1995) appear to have been performed according to standard protocols. No induction of SCEs was observed in tests involving up to 50ug/mL PH with or without S9 mix. At this dose level cytotoxicity was evident. In the same test system no chromosomal aberrations were seen in response to PH in the absence of S9 mix. However, in response to the cytotoxic 50ug/mL dose level in the presence of S9, but not in response to 30ug/mL, 23ug/mL or 11ug/mL in the presence of S9, chromosonal abnormalities were seen.

I find it surprising that the Draft NTP Report does not draw attention to the fact that positive results only occurred at cytotoxic concentrations in these studies.

14.3 The protocols for the in vivo studies of the effect of PH on frequency of micronuclei were unusual insofar as animals were exposed to PH for 13 weeks before counts were made. The number of control mice was low and there is inadequate background information from tests on other chemicals where there has been exposure over periods of 13 weeks. It seems that Witt et al (1995) observed increased micronuclei in response to PH in $B6C3F_1$ mice but only under conditions of very high dosage.

I strongly recommend that a further *in vivo* micronucleus test of conventional design with exposure to realistic dose levels be carried out.

I note that Dietz *et al* (1992) carried out a 13-week study in F344 rats as well as a study of similar duration on $B6C3F_1$ mice. Did they do micronucleus counts on the rats as well as on the mice? If so, what did they find?

15. The materials and methods used in the carcinogenicity studies in rats and mice

15.1 The purity of the 3 different lots of PH used for the animal studies was analysed using a variety of analytical techniques. The overall purity of all three lots was equal to, or better than, 98%. Stability studies indicated that under the conditions of storage in the dark at about 25°C, as were used during the performance of the studies, no degradation took place. It is likely that the purity of the PH to which the animals were exposed was as high as, or higher than, that of the PH incorporated into laxative formulations for human use.

15.2 The concentrations of PH in the diet formulations given to the rats and mice in the NTP studies were checked and with only one, rapidly corrected, exception found to be within 10% of the target range.

15.3 14-day studies (carried out during 1979)

After checks had been made that animals were free from parasites and of gross evidence of disease groups of 5 male and 5 female F344/N rats and of 5 male and 5

female B6C3F$_1$ mice were fed, ad libitum, on diets containing 0, 6,250, 12,500, 25,000, 50,000 or 100,000 ppm PH for 14 days with water also being provided ad libitum. Animals were housed 5 per cage. Clinical observations were recorded daily, and food consumption on days 7 and 14. Animals were weighed initially, on day 7 and at the end of the study. Gross necropsies were carried out on all animals but histopathological examination was limited to 3 control rats, 3 control mice and 3 mice exposed to 100,000 ppm PH. The source of the animals used in this study was different from that of the animals used in the 13 week and 2 year studies.

15.4 13-week studies (started in 1987)

A primary aim of these studies was to determine the dose levels to be studied in 2-year studies. Groups of 10 male and 9 or 10 female rats, and similar sized groups of mice, were fed ad libitum on diets containing 0, 3,000, 6,000, 12,000, 25,000 or 50,000 ppm. Rats were housed 5 per cage and mice 1 per cage. Animals were observed daily, weighed once weekly and clinically examined once weekly. Food consumption was measured weekly. At the end of the studies all animals were necropsied and the following organs were weighed: brain, heart, right kidney, liver, lung, right testis and thymus. Blood samples were collected terminally for haematological and clinical chemistry measurements. Additional groups of 10 animals of both species/sex/group were included for haematology and clinical pathology. At the end of the studies, complete histopathology was carried out on animals in the control and 50,000 ppm group. At the end of the study sperm samples were collected from the 0, 12,000, 25,000 and 50,000 ppm groups. Also vaginal fluid

samples were collected from female rats and mice from the control, 12,000, 25,000 and 50,000 ppm groups for evaluation with regard to the frequency of oestrous stages and oestrous cycle lengths.

15.5 The two-year studies in F344 and B6C3F$_1$ mice (started in 1991)

In the 2-year studies groups of 50 animals/sex were fed ad libitum on diets containing 0, 12,000, 25,000 or 50,000 ppm PH in the case of rats and 0, 3,000, 6,000 or 12,000 ppm PH in the case of mice. Rats were housed 5 per cage and mice 1 per cage. During the study animals were observed twice daily. Clinical findings were recorded every 4 weeks, body weights were recorded initially, then once weekly for 13 weeks, and thereafter once every 4 weeks. Blood samples were taken at the termination of the study and analysed for plasma concentration of PH. No clinical pathology or haematological observations were made either during the study or terminally. A complete necropsy and microscopic examination was performed on all rats and mice. The histopathological findings were checked by a quality assurance pathologist and submitted to a NTP Pathology Working Group. Results were analysed statistically by the use of standard techniques.

16. Results of 14-day studies

16.1 In the 14-day rat study all the animals survived until the end of the study. Treated animals of both sexes and at all dose levels put on slightly but not significantly less weight than the controls. Top-dose (100,000 ppm PH in the diet) animals of both sexes ate more food than the controls. The estimated intakes of

PH ranged from 500 to 10,500 mg/kg bw/day in males and from 500 to 11,000 mg/kg bw/day in females. The faeces of top-dose males and females were paler than those of animals in other groups.

16.2 No animals died during the 14-day mouse study and food consumption was slightly higher in all treated groups than in controls. However, the difference in food consumption was not associated with any corresponding effect on body weight gain. All the animals remained clinically normal and their faeces appeared normal.

17. Results of 13-week studies

17.1 All the animals in the 13-week rat study survived until the end of the study. Body weight gain was reduced, but not significantly so, in males given 25,000 or 50,000 ppm PH in the diet and was significantly ($p<0.05$) reduced in females at these same two dose levels. Absolute and relative liver weights were significantly higher in males and females exposed to 25,000 ppm or 50,000 ppm PH in the diet and the same is true for relative liver weight in males receiving 12,000 ppm PH in the diet.

The findings in the study point to the liver being a possible target for toxicity in response to PH in the rat.

17.2 In the 13-week mouse study, all the animals survived until the end of the study. Weight gain was slightly, but not significantly, lower and food consumption was slightly, not not significantly higher, in top-dose (50,000 ppm PH in the diet) animals of both sexes than in controls. Exposure of males to 12,000, 25,000 or 50,000 ppm was associated with reduced epididymis

weight in males and animals exposed to 12,000 or 50,000 ppm showed significantly reduced sperm counts. Absolute and relative testis weight were both lower in all groups receiving diets containing 6,000 ppm or more PH compared with controls, and absolute testis weight was significantly less in males given the 3,000 ppm PH diet than in those given the control diet. These changes in testicular weight were associated with the loss of one or more generations of germ cells and other cellular changes. Significantly increased incidences of bone marrow hyperplasia and necrosis were seen in almost all males and females exposed to 12,000 ppm or more PH in the diet, whilst significantly increased haematopoiesis was seen in males exposed to 25,000 or 50,000 ppm PH in the diet.

The effects of PH on the testis and on sperm counts in mice suggest that PH possesses oestrogenic activity.

18. Results of the 2-year rat study

18.1 The dose levels selected for testing in the 2-year rat study were 0, 12,000, 25,000 and 50,000 ppm PH in the diet. This design excluded the 3,000 ppm and 6,000 ppm dose levels which were found to be NOELS for effects on the liver in the 13-week rat study. Survival was not adversely affected at any of the 3 dose levels tested in either sex. Feed consumption was not affected by PH but body weight gain was slightly reduced (e.g. by up to about 15%) in a dose-related fashion in all treated groups in both sexes. The only clinical finding noted was ruffled fur in males of all exposed groups. In this study, exposure to PH in males amounted to approximately 500, 1,000 and 2,000 mg/kg bw PH and that in females to approximately 500, 1,000 and 2,500 mg/kg bw PH.

Plasma levels of total PH (free and conjugated) in blood samples taken at 5 different time points on the last day of the study showed no consistent pattern with respect to time of day and relatively little between-group difference despite the 4-fold difference in daily oral dose between the low dose (12,000 ppm) and the high dose (50,000 ppm) groups.

18.2 The following incidences (%) of neoplasms were reported in MALE rats :

PH ppm	0	12,000	25,000	50,000
<u>Adrenal medulla</u>				
- Benign phaeochromocytoma	34	68^{+++}	68^{+++}	68^{+++}
- Benign or malignant phaeochromocytoma	36	70^{+++}	70^{+++}	70^{+++}
<u>Kidney</u> (Single & step sections)				
- Renal tubule adenoma	2	20^{+++}	30^{+++}	30^{+++}
- Renal tubule adenoma or carcinoma	2	20^{+++}	32^{+++}	32^{+++}
<u>All organs</u>				
- Benign neoplasm	94	100	98	100
- Malignant neoplasm	70	72	62	66
- Benign or malignant neoplasm	98	100	100	100

18.3 The following incidences (%) of non-neoplastic lesions were reported in MALE rats :

PH ppm	0	12,000	25,000	50,000
Adrenal medulla				
Focal hyerplasia	24	42	32	38
Focal hyperplasia, bilateral	2	2	4	8
Parathyroid gland				
Hyperplasia diffuse	0	13	6	17
Hyperplasia diffuse, bilateral	0	21	22	13
Adenoma	0	1	0	0
Forestomach				
Chronic inflammation	0	0	10	4
Ulcer	4	4	8	6
Epithelial hyperplasia	8	4	24	18
Glandular stomach				
Mineralization	0	22	10	12
Heart				
Myocardial mineralization	0	0	4	2
Aorta				
Mineralization	0	18	6	8
Kidney				
Hyperplasia of transitional epithelium in pelvis	8	62	68	58
Femur				
Fibrous osteodystrophy	0	34	28	24

Key +++ = p<0.001
++ = p<0.01

18.4 The following incidences of neoplasms were reported in FEMALE rats (%):-

PH ppm	0	12,000	25,000	50,000
Adrenal medulla				
- Benign phaeochromocytoma	6	22	18	4
- Benign or malignant phaeochromocytoma	6	24+	20+	4
All organs				
- Benign neoplasm	86	98	84	82
- Malignant neoplasm	60	50	42	46
- Benign or malignant neoplasm	98	100	100	98

18.5 The following incidences of non-neoplastic lesions were seen in FEMALE rats (%):-

Adrenal medulla				
Focal hyperplasia	18	28	30	22
Focal hyperplasia, bilateral	2	8	0	0
Parathyroid gland				
Focal hyperplasia	2	2	2	0
Aorta				
Mineralization	2	0	0	0
Kidney				
Hyperplasia of transitional epithelium of pelvis	0	2	2	0
Femur				
Fibrous osteodystrophy	2	0	0	0

18.6 Interpretation of the findings in the 2-year rat study

18.6.1 The first thing to note is that the suspicion based on the findings in the 13-week study (see Section 17.1) that the Liver might be a target for toxicity, was not borne out in the 2-year study when the only between-group difference of note was increased cytoplasmic vacuolation of hepatocytes in treated males of all groups as compared with control males.

No dose ended

18.6.2 More importantly, there was, in males, evidence of a syndrome of changes that are known from previous studies to be associated with a disturbance of calcium metabolism (Roe, 1993; ANNEX B). The list of changes so associated includes:-

(i) Increased incidence of hyperplasia of the adrenal medulla,
(ii) Increased incidence of benign and malignant phaeochromocytoma,
(iii) Increased incidence of parathyroid hyperplasia,
(iv) Increased incidence of mineralization in tissues such as the glandular stomach, aorta, and myocardium,
(v) Fibrous osteodystrophy,
and,
(vi) Hyperplasia of the transitional epithelium of the renal pelvis.

18.6.3 It is usual for males to be more susceptible to develop these changes than females. Deposition of minerals in the pelvic space of the kidney (i.e. pelvic mineralization) commonly accompanies the above changes and where it does so, may be relevant to the development of hyperplasia of the pelvic epithelium. In the present study, pelvic mineralization was not reported but this may have been because the pathologist who read the study, did not regard it as a pathological change.

18.6.4 The syndrome of changes is associated with hypercalcaemia. Unfortunately blood calcium levels were not measured in the 13-week rat study and further studies are now needed to see if exposure to high doses of PH give rise to continuous or intermittent

hypercalcaemia in male rats.

18.6.5 Two causes of hypercalcaemia that give rise to the syndrome in male rats have been described (Roe, 1993).

18.6.6 The first is chronic progressive nephropathy which occurs spontaneously and in increasing severity and incidence in male rats, especially if they are overfed. In the present study, no attempt was made by the pathologist to record the severity of chronic nephropathy: it was simply recorded as present in 90% of control males and in between 96% and 100% of males in the 3 treated groups. Hence it is not possible to be sure whether exposure to PH affected the severity of this age-related change.

18.6.7 The second mechanism known to be associated with some of the manifestations of the syndrome constitutes a response to high oral doses of lactose, various polyols (e.g. sorbitol, xylitol), and certain chemically-modified food starches. Heavy consumption of these carbohydrates is associated with increased adrenal meduallary hyperplasia and neoplasia, increased pelvic nephrocalcinosis and hyperplasia of the transitional epithelium of the renal pelvis, hyperplasia and neoplasia of the parathyroid gland and metastatic calcification in various tissues. The underlying cause of these changes is increased absorption of calcium from the intestinal tract (Hodgkinson et al 1982 [ANNEX C]; Roe 1993 [ANNEX B].

18.6.8 In the light of the second of these known mechanisms, I regard it as highly likely that lesions listed as (i) to (vi) in section 18.6.2 are all manifestations of disturbed calcium homeostasis. And, if this is true, then the effect of heavy oral

exposure to PH on the incidence of adrenal medullary neoplasms in the male rats, can be explained in terms of a previously described non-genotoxic mechanisms.

18.6.9 One possible explanation of the increased incidence of renal adenomas in PH-treated male rats is that PH induces alpha$_{2u}$-globulin nephropathy which is known to progress to renal adenoma development. Although it is unlikely that an effect of PH on this form of nephropathy was overlooked in the 13-week study, it is not impossible that this could have happened in a study conducted in 1987. This being so, it would be worthwhile for the kidney sections from the males in the 13-week rat study to be reviewed, particularly since no increased incidence of renal tumours was seen in female rats exposed to PH. In the Discussion section of the NTP report, it is hypothesised that increased cell turnover in renal tubule cells secondary to chronic progressive nephropathy might predispose to adenoma development.

18.6.10 In males, the high incidence of chronic inflammation and epithelial hyperplasia in the forestomach is indicative of no more than that PH is somewhat irritant to forestomach epithelium.

18.6.11 In contrast to the findings in males, there was no histopathological evidence of disturbance of calcium homeostasis in females exposed to PH. This is not surprising in so far as in the studies with lactose and the polyols female rats were found to be very much less sensitive than males to this manifestation of toxicity (see Roe 1993; ANNEX B).

18.6.12 Similarly, in relation to the absence of any effect of exposure to PH on the incidence of renal adenomas,

it is noteworthy that females are not susceptible to alpha$_{2u}$-globulin nephropathy.

18.6.13 Despite the increased incidences of adrenal medullary tumours and renal adenomas in PH-treated males, it is notable that the overall incidences of benign and/or malignant neoplasms at all body sites was not adversely affected by exposure to PH in either sex (see 18.2 and 18.4).

19. Results of the 2-year mouse study

19.1 The dose levels selected for testing in the 2-year mouse study were 0, 3000, 6000 and 12000 ppm. This design excluded the two highest dose levels tested in the 13-week test. Survival was lower in all treated groups than in controls in the case of both sexes. However, only in the case of top-dose females was the difference statistically significant. Body weight gain was slightly (up to 9%) lower in all treated groups of both sexes than in controls, the reduction being most marked in top-dose females. On the other hand, PH did not affect feed consumption. The doses of PH consumed by males were approximately 300, 600 and 1200 mg/kg bw/day and those by females approximately 400, 800 and 1500 mg/kg bw/day. However, at the end of the study, plasma levels in top dose animals were only marginally, and not signficantly, higher in top dose animals than in the controls. No clinical effects were observed in PH-exposed mice.

19.2 The following incidences (%) of neoplasms were seen in MALE mice:-

PH ppm	0	3000	6000	12000
Histiocytic sarcoma (all organs)	2	6	22^{+++}	24^{+++}
Malignant lymphoma (all organs)	12	16	24	16
All neoplasms (all organs)				
- Benign	58	48	36^{-}	39^{-}
- Malignant	38	58^{+}	70^{+++}	59^{+}
- Benign or malignant	80	80	84	78

Key +++ = $p<0.001$
++ = $p<0.01$
+ or - = $p<0.05$

19.3 The following incidences (%) of non-neoplastic lesions were reported in male mice:-

PH ppm	0	300	600	12000
Bone marrow				
- myelofibrosis	6	16	16	39^{++}
- pigmentation	0	4	10^{+}	33^{++}
Testis				
- degeneration of germinal epithelium	2	98^{++}	100^{++}	98^{++}
Thymus				
- atypical hyperplasia	0	7	16	17
Kidney				
- renal tubule hyaline droplets	0	4	10^{+}	20^{+}

Key as for neoplasms

19.4 The folloiwng incidences (%) of neoplasms were observed in FEMALE mice:-

PH ppm	0	3000	6000	12000
Liver				
- Adenoma	34	4^{---}	12^{---}	2^{---}
- Carcinoma	12	2^{---}	0^{---}	2^{---}
Ovary				
- Benign sex-cord stromal tumour	0	14^{++}	12^{+}	10^{+}
Histiocytic sarcoma (All organs)	0	4	14^{++}	14^{++}
Malignant lymphoma (All organs)	30	56^{++}	66^{+++}	50^{+}
All neoplasms (all organs)				
- Benign	52	40	46	34
- Malignant	50	68	82	74
- Benign or malignant	80	78	94	86

Key +++ or --- = $p<0.001$
++ or -- = $p<0.01$
+ or - = $p<0.05$

19.5 The following incidences (%) of non-neoplastic lesions were reported in FEMALE mice:-

PH ppm	0	3000	6000	12000
Liver				
- Eosinophilic focus	40	8^{--}	4^{--}	1^{--}
Heart				
- Mineralization	0	10^{+}	8^{+}	14^{+}
Thyroid				
- Follicular cell hyperplasia	54	16^{--}	6^{--}	14^{--}
Ovary				
- Hyperplasia	6	20^{+}	20	34^{++}
Bone marrow				
- Pigmentation	4	6	22^{++}	22^{++}
Thymus				
- Atrophy	6	2	12	20
- Atypical hyperplasia	0	16	12	11
Kidney				
- Renal tubule hyaline droplets	0	2	6	16^{++}

Key as for neoplasms

19.6 Intepretations of the findings in the 2-year mouse study

19.6.1 Many of the effects seen in the 2-year mouse study can be explained by the previously reported weak oestrogenic effect of PH (Bitman and Cecil, 1970; Nieto et al, 1990). This is true for the adverse effects on the incidence of histiocytic sarcoma and malignant lymphoma in both sexes; the non-neoplastic effects on the testis, ovary, thymus and bone marrow (myelofibrosis) in males; and the effect on ovarian stromal tumour in females. Gardner (1941) and subsequently many other investigators, reported increased incidences of malignant lymphoid neoplasms in resonse to oestrogens in mice, but in the earlier

reports no clear distinction was made between malignant lymphoma and histiocytic sarcoma. There is no doubt, in my opinion, that the incidence of both these kinds of neoplasm is enhanced by oestrogenic activity and that the mechanism responsible involves hyperplasia of the thymus gland. Testicular atrophy is to be expected as a response to oestrogenic activity and it is very likely that the increased incidence of sex-cord stromal tumours is a further manifestation of disturbed hormonal balance.

19.6.2 In view of the findings in rats, it is interesting that a slightly higher incidence of mineralization was seen in the heart in treated females.

19.6.3 The slightly increased incidence of renal tubular hyaline droplet accumulation in the kidneys of mice of both sexes remains unexplained as does the highly significant reduction in thyroid follicular cell hyperplasia in females. However, the latter could well be due to reduced pituitary thyrotropin production secondary to oestrogenization.

19.6.4 Two somewhat surprising findings were the highly significant reductions in incidences of liver cell adenoma, adenocarcinoma and eosinophilic liver foci in females. These findings are surprising since in the past there has been a concern about increased liver-tumour incidence in response to contraceptive pill formulations. However, the latter have gestogenic as well as oestrogenic components and it is now widely recognized that the gestogenic components are the more important in relation to liver tumour risk.

20. Discussion section of Draft NTP Report in relation to results of 2-year studies

20.1 The report points out that the PH molecule contains a triphenylmethane structure which may act as an oestrogen agonist or antagonist through interaction with the oestrogen receptor protein (Ravdin et al, 1987).

20.2 On page 106 of the draft NTP Report, it is stated that the incidence of chronic nephropathy was higher in female rats exposed to PH than in control females and that the severity of this ageing related change was higher in PH-exposed males than in control males. These findings may well be relevant to the treatment related increase in incidence of adrenal medullary proliferative changes and of other manifestations of disturbed calcium homeostasis (see section 18.6.6).

21. Relationship between findings in tests of PH for genotoxicity and findings in 2 year studies

It is pointed out in the Draft NTP report that it is unusual for an agent which produces chromosomal breaks not also to give rise to sister chromatid exchanges. For this and other reasons it seems that the genetic damage caused by PH is very specific.

22. Comments on doses selection for testing in the 2-year studies

22.1 Daily dosages for adult humans range from 30 to 270 mg, equivalent to 0.5 to 4.5 mg/kg bw per day.

22.2 The dosages given to male rats in the 2-year rat study approximated to 500, 1000 and 2000 mg/kg bw/day and the doses given to female rats approximated to 500, 1000 and 2500 mg/kg bw/day (see paragraph 18.1). Thus, the rats, even in the lowest of the 3 dose levels studied, were exposed to more than 100 times the highest usual human daily dose.

22.3 In response to these dose levels there was a major disturbance of calcium homeostasis together with enhancement of the incidence/severity of chronic progressive nephropathy and these effects can account for all the pathological changes including the adrenal and renal tumours seen in excess in males. In females, despite the heavy dosage, there were no adverse effects on tumour incidence or other pathology.

22.4 In other words, non-genotoxic carcinogenic mechanisms can account for all the adverse effects on tumour incidence seen in rats exposed to very high doses of PH.

22.5 The dosages of PH given to male mice in the 2-year mouse study were approximately 300, 600 and 1200 mg/kg bw/day and those in females were approximately 400, 800 and 1500 mg/kg bw/day. Thus, the mice in the lowest of the 3 dose levels studied were exposed to between 67 and 89 times the highest usual human daily dose.

22.6 In response to these dose levels a range of effects were seen on the incidence of neoplastic and non-neoplastic changes, virtually all of which can be attributed to the known oestrogen agonist/oestrogen

antagonist activity of PH brought about by its interaction with oestrogen receptor protein (Ravdin et al, 1987).

22.7 Thus in mice as in rats all the adverse effects on both neoplastic pathology and non-neoplastic pathology are explicable in terms of non genotoxic carcinogenic mechanisms.

22.8 Unfortunately, the design of the 2-year studies conducted NTP was not suited to demonstrating NOEL's for non-genotoxic carcinogenic mechanisms and some of the effects seen were evident at the lowest dose level tested (e.g. the effects on calcium homeostasis in male rats and the effects on the testis, ovary and malignant lympohoma in mice). Therefore, further studies will have to be undertaken at realistic dose levels in order to establish safety margins. It is noteworthy that 1000 ppm PH in the diet was a NOEL for all the parameters measured in a continuous breeding study in Swiss CD-1 mice (see Appendix N of NTP Report). The parameters measured included litter size, weight, survival, reproductive performance, length of gestation, sperm parameters, oestrous cycle, characteristics, etc.)

22.9 Instead of accepting that the increased tumour incidences seen in the 2 year rat and mouse studies were manifestations of non-genotoxic carcinogenicity the authors of the Draft NTP Report seem to have chosen to interpret the extremely odd findings in various non-standard short term tests as indicative of genotoxicity and to relate these odd findings to the effects on tumour incidence.

23. Opinion and Recommendation

23.1 I strongly suspect that most of the adverse findings in the 2 year rat study are attributable to an effect of PH on calcium homeostasis and that most of the adverse findings in the 2-year mouse study are attributable to its known oestrogenic/anti-oestrogenic activities.

23.2 It is very probable that NOEL's for these effects which allow ample safety margins could be established.

23.3 A specially designed study in male rats is likely to confirm the effect of PH on calcium homeostasis in male rats. The study reported by Hodgkinson et al (1982) relating to lactose and chemically modified starches, is relevant in this regard.

23.4 In recent years it has been increasingly widely recognised that the results of tests for carcinogenicity involving the exposure of animals to chemicals at maximum tolerated dose levels (MTD), in the absence of information on the response of animals to realistic dose levels of the same chemicals, can be difficult to interpret and lead to false conclusions. The studies of NTP exemplify this situation.

23.5 Such relevant epidemiological evidence as exists, although not very helpful, does not point to there being any carcinogenic risk from exposure to PH.

Signed:.....

Francis J C Roe

DM, DSc, FRCPath, FATS

Date: 8th February, 1996

References

Bitman J and Cecil HC (1970) Estrogenic activity of DDT analogs and polychlorinated biphenyls
J. Agric. Food Chem., 18: 1108-1112

Dietz DD, Elwell MR, Chapin RE et al (1992) Subchronic (13-week) toxicity studies of oral phenolphthalein in Fischer 344 rats and B6C3F$_1$ mice
Fundam. Appl. Toxicol., 6: 44-52

Federal Register (1975) Food and Drug Administration Over-The-Counter Drugs: Proposal to establish monographs for OTC laxative, antidiarrheal, emetic and anti-emetic products Vol 40: 12901-12944

Galloway SM, Armstrong MJ, Reuben C et al (1987) Chromosome aberrations and sister chromatid exchanges in Chinese hamster ovary cells: Evaulations of 108 chemicals
Environ. Mol. Mutagen, 10 (Suppl 10): 1-175

Gardner WU (1941) The effect of oestrogen on the incidence of mammary and pituitary tumours in hybrid mice
Cancer Res., 1: 345-358

Haddow A and Horning ES (1960) On the carcinogenicity of an iron-dextran complex
J. Natl. Cancer Inst., 24: 109-147

Hodgkinson A, Davis D, Fourman J, Robertson WG and Roe FJC (1982) A comparison of the effects of lactose and of two chemically modified waxy maize starches on mineral metabolism in the rat
F Chem. Toxicol., 20: 371-382

Kune GA (1993) Laxative use not a risk for colorectal cancer: Data from the Melbourne-Colorectal-Cancer-Study
Z. Gastroenterol, 31: 140-143

Mortelmans K, Haworty S, Lawlor T et al (1986) Salmonella mutagenicity tests: II Results from the testing of 270 chemicals
Environ. Mutagen., 8 (Suppl 7) : 1-119

Nieto A, Garcia C and Lopez de Haro MS (1990) In vivo estrogenic and anti estrogenic activity of phenolphthalein and derivative compounds
Biochem. Int., 21: 305-311

Ravdin PM, Van Beurden M and Jordan VC (1987) Estrogeic effects of phenolphthalein on human breast cancer cells in vitro
Breast Cancer Res. Treat., 9: 151-154

Witt KL, Gulati DK, Jaur P and Shelby MD (1995) Phenolphthalein: Induction of micronucleated erythrocytes in mice
Mutat. Res., 341: 151-160

Wu AH, Paganini-Hill A, Ross RK and Hendeson BE (1987) Alcohol, physical activity and other risk factors for colorectal cancer: A prospective study
Br. J. Cancer, 55: 687-694

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National Toxicology Program
Report on Carcinogens Group

Roe FJC. 1993. Mineral metabolism and toxicity. In: Parke DV, Ioannides C, Walker, R, editors. Food, Nutrition and Chemical Toxicity. London: Smith-Gordon and Company Limited. p. 91-104.

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FAX TO: Francis Roe

From: Christopher Kemp
Assistant Member
Program in Cancer Biology
Division of Public Health Sciences

Dat Correspondence with Dr. Kemp

Number of pages:1
Dear Dr. Roe,
Thanks you for your fax dated 10 June 1996. I am very interested in your questions as I have been interested in chemical regulation and carcinogen bioassays for many years. However, I am not associated with any regulatory agency and so can only give my opinion. I am not sure what changes if any will be made with regard to the use of p53 null or other transgenic models. I think the best approach is to continue to experiment with these models in order to learn more of the mechanism of carc. For example one chemical might enhance tumorigenesis in p53 null mice but another might do in a H-ras transgenic mouse. These types of experiments will shed light on the mechanism. Then if for example a substance is found to only act as a promoter, it might be regulated differently then one found to act by initiating or mutagenesis. If a substance is found to be carcinogenic in the p53 null mice then I believe it should carry some regulatory weight, that is to say I would be in favour of regulating this compound. This is true even if the compound were negative in wild type mice. The reason is that loss of p53 is common in human tumors and so presumably , this chemical could accelerate the appearance, growth, or malignant progression of a pre-existing p53 mutated tumour. However, the p53 null mice develop tumors spontaneously at such a high rate that I think it will be unlikely that many chemicals will be able to accelerate this. It may not be possible to detect any effect. I think a better model is the p53 heterozygotes which develop tumors much later, it is easier to detect acceleration of latency and one can also examine the remaining wild type allele for mutation.

Your second question, is no I am not positive that the carcinomas from the p53 null mice arose from papillomas. I guess it is somewhat a matter of semantics. as their may have been benign precursor cells to the carc. but not visible as a papilloma.

Another contact and friend of mine who is involved in regulation and p53 transgenic models is

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NIEHS
PO box 12233
Research Triangle Park
NC USA 27709-2233
FAX 919 541 1460

Thanks for your interest

Sincerely

Chris Kemp

(Attached)

Carcinogenicity Assessment Committee
April 2, 1996

Attendees: See Attachment

Dr. DeGeorge, Acting Chair, began the meeting with a description of the CAC process for reviewing drug products. Dr. DeGeorge explained to the meeting attendees that animal tumor findings do not always equate to human risk. Consideration must be given to tumor types, background incidence, and study design and clinical use pattern. The National Toxicologic Program (NTP) nominated phenolphthalein as a candidate for carcinogenicity testing due to the widespread use and lack of carcinogenicity data. The draft report on phenolphthalein was presented to the NTP's Board of Scientific Advisors in December 1995. He explained that NIEHS/NTP had begun conducting additional studies to further assess the carcinogenic/genotoxic potential of phenolphthalein. However, due to the federal shut down, completion of these studies was delayed. Therefore, the data are not available for consideration at this time. The NTP will provide detailed information on these studies during their presentation.

Dr. Bowen provided a brief description of the 1985 tentative final rule for over-the-counter laxative drug products including the mechanism of action and clinical usage. She elaborated on the clinical use and misuse of over-the-counter (OTC) laxative products. Dr. Bowen indicated that the findings reported by NTP are of particular concern for the chronic intermittent use of phenolphthalein-containing products.

National Toxicological Program Presentation
Dr. Bucher presented a summary of NTP's interpretation of the study findings addressing the carcinogenic and genotoxic potential of phenolphthalein. The NTP found phenolphthalein negative in the standard *Salmonella* assay both with and without metabolic activation. However, phenolphthalein was positive for *in vitro* chromosomal aberrations and Chinese hamster ovary (CHO) cells. Phenolphthalein also induced micronuclei in peripheral red blood cells and bone marrow in mice when treated in long term studies. The NTP concluded that phenolphthalein is a multisite carcinogen in both mice and rats and presents some risk to humans based on the reported DNA damage with in vivo and in vitro tests, the estrogenic/antiestrogenic effects, and the oxidative effects of the drug.

Dr. Dunnick, Study Scientist, presented an overview of the NTP findings: pheochromocytomas in male and female rats, renal tumors in male rats, histiocytic sarcomas in mice, thymic lymphomas in mice, and stromal tumors in female mice. She reported that phenolphthalein is the only chemical of 200 tested at NTP to produce adrenal tumors in both sexes of the rat. The incidence of renal lesions was outside the historical control range only in the LD and HD male rats. Step sectioning to investigate these tumors in female rats yielded no significant findings. The hematopoietic

Annex C

tumors were reported in all dose groups of the male and female mice including controls. Significant increases were found in the MD and HD groups for both males and females for the histiocytic sarcomas. The malignant lymphomas reported in the mouse study were increased significantly only at the MD for males, but significantly increased in females for LD, MD, and HD groups. These tumors are believed to be of thymic origin. Dr. Dunnick commented that lymphomas of thymic origin have been reported in only 2 other compounds tested by NTP and consider this tumor type to be rare. The ovarian tumors reported in the female mice were significantly increased compared to controls for all dose levels.

The NTP concluded from the study findings that in male rats there is clear evidence of carcinogenic activity for the adrenal medulla pheochromocytomas and renal tubule neoplasms; in female rats there is some evidence of carcinogenic activity for the adrenal medulla pheochromocytomas; in male and female mice there is clear evidence of carcinogenic activity for the histiocytic sarcomas and malignant lymphomas of thymic origin; and in the female mice there is clear evidence of carcinogenic activity for the ovary tumors (see Appendix A for NTP definitions of carcinogenic activity).

Dr. Dunnick also described the additional studies currently being conducted by the NTP to further evaluate phenolphthalein:

1. Mechanism of action - study cell damage brought on by diphenylmethanes and triphenylmethanes through the oxidation and redox cycling and subsequent DNA damage by measuring hydroxyl radical DNA-adducts.

2. Estrogenic/antiestrogenic activity - being measured using three tests: estrogen receptor competitive binding assay, uterotropic bioassay, and transcriptional activation in ER-transfected HELA calls.

3. Transgenic studies - P53 hemizygous female mouse using the high and low dose from the carcinogenicity study, as well as three lower dose levels similar to human dose levels on a mg/m2 body surface area basis.

4. Molecular biology studies - evaluating changes in p53 gene expression in several of the tumors found in the bioassay.

5. Toxicokinetic and distribution studies: toxicokinetic studies have been conducted in rats and mice to develop a model to predict tumor incidences at lower dose levels. The lower doses are comparable to human doses on a mg/m2 basis.

Dr. Hailey, Study Pathologist, provided background incidence of the tumor types reported in mice with phenolphthalein relative to NTP's experience. Histiocytic sarcomas are not commonly seen within NTP studies, 0-2% in male untreated controls and 0-4% in female untreated controls. The NTP concluded that the increased incidence of these tumors, given the rare nature of this tumor, it

is unlikely that the rates observed in this study could occur by chance. The background incident rate for malignant lymphomas is 23% in females and 9% in males. In the past, malignant lymphomas have often included the category of lymphomas for thymic origin because of the difficulty in site of origin determination. The NTP believes histopathologic data support that the lymphomas reported in this study are of thymus origin. Eight additional pathology experts in rodent toxicology, reviewed some or all of the lesions and agreed that these were thymic in origin and a treatment-related effect. Regarding the ovarian lesions, Dr. Hailey explained that there are three categories: germ cell, epithelium or sex-cord stromal. The ovarian were considered sex-cord stromal in origin by the expert rodent toxicologic pathologists.

Sandoz Presentation

Dr. Lapadula presented the findings for Sandoz Pharmaceuticals genotoxicity and metabolism studies (all study results were presented in draft since the data have not yet been audited). The firm believes, based on their study findings, that the genotoxicity reported in rodents was present only at saturation of exposure levels and the tumor findings may be related to nongenotoxic mechanisms. Sandoz found CHO cells were positive in the presence of induced and noninduced rat S9 and with human S9 and negative in the presence of induced and noninduced rat hepatocytes and with human hepatocytes for chromosomal aberrations. Human lymphocytes were negative in the presence of human S9 metabolic activation and equivocal in the presence of noninduced rat S9 metabolic activation for chromosomal aberrations. Both CHO cells and human lymphocytes were negative in the absence of S9 metabolic activation. The firm believes that interpretation of these findings suggest the parent compound, phenolphthalein, is not genotoxic. They suggest that the oxidative metabolites formed at high cytotoxic concentrations of drug may be responsible for the genotoxicity. In addition, the human and rodent hepatocyte results suggest that whole cell systems may be capable of protecting from the genotoxicity. Further, the human cells appear to be less sensitive to genotoxicity based on the human lymphocyte results.

Sandoz reviewed positive findings for the 13-week in vivo mouse micronucleus NTP study. Although positive findings were reported in doses at 6000 ppm and higher, the AUCs indicated that the saturation of exposure was reached at a dose of 3000 ppm and higher. The AUC of the lower doses in rodents was 30-80 times greater than the labeled human dose of 150 mg. Dr. Lapadula concluded that micronuclei were generated at doses that saturate exposure and again suggested that the clastogenic activity detected was associated with drug metabolism at toxic exposures.

The preliminary in vitro metabolism data generated by Sandoz with microsomal fractions suggested that the rat, mouse, and human are all capable of producing the same metabolites. However, the data suggest that humans are less efficient (5- to 6-fold) at producing oxidative metabolites and are very efficient at detoxication of phenolphthalein and metabolites produced through glucuronidation.

Dr. Lapadula hypothesized that nongenotoxic mechanisms, alteration of calcium homeostasis and estrogenic activity, may account for the tumors observed in the bioassays conducted by NTP.

Consideration was given to known toxicities associated with alteration of calcium homeostasis, findings also reported in the rat bioassay. Other studies have reported phenolphthalein alters calcium homeostasis in rat and human in vitro tissue preparations. Estrogenic activity of a compound can result in malignant lymphomas/histiocytic sarcomas, ovarian stromal tumors, and non-neoplastic changes in testes, ovary, thymus, and bone marrow, similar to those reported in the mouse bioassay.

Dr. Gelbert concluded Sandoz's presentation by summarizing the salient points supporting the lack of evidence for genotoxicity in human cells and the possible nongenotoxic mechanisms of tumor formation.

FDA Presentation

Dr. Choudary presented the Division of Gastrointestinal and Coagulation Drug Products' review and interpretation of the NTP findings. For the rat carcinogenicity study, he considered the achieved dose levels (500, 1000, and 2000 mg/kg) to be excessive based on the plasma concentration achieved and reported toxicities. AUCs for the rat were 50-100 fold the normal human clinical exposure. While body weight decreases for treated animals were 15-21% in males and 13-15% in females, well above the recognized 10% decrease for MTD. The reported tumors, adrenal medulla (benign and malignant pheochromocytomas) and renal tumors were considered to be the consequence of toxicity inflicted with excessive doses rather than clear evidence for carcinogenic activity. Decreased body weights and increased incidence in nephropathy further supported the excessive dosing and toxicity-related tumors.

For the mouse carcinogenicity study, Dr. Choudary found the achieved dose levels (300, 600, and 1200 mg/kg in males and 400, 800, and 1500 mg/kg in females) also to be excessive. The reported AUCs in mice were 70-100 fold that of normal human clinical exposure. He considered the reported histiocytic sarcomas a result of the bone marrow toxicity produced by the excessive doses used in the study, particularly for the males since other hematopoietic-related tumors (malignant lymphomas) were within historical control. The malignant lymphomas were significantly increased in females at all dose levels. Dr. Choudary questioned the NTP's use of a recently developed diagnostic classification for the hematopoietic system, bringing into question the narrow historical control range used for comparisons as well as the actual tumor classification. The increased incidence of tumors of the hematopoietic system were probably due to the specific toxic lesions inflicted on the primary site of hematopoiesis (i.e., bone marrow). In his opinion, the "ovarian tumors" may not be a tumorigenic response, but represent hyperstimulated interstitial cells organized into pseudoglandular structures under the influence of hormonal interplay on the ovarian-hypophysial hypothalamic axis. The observed proliferative lesions in this study probably do not constitute clear evidence for carcinogenicity.

General Discussion

Adrenal Medulla Pheochromocytomas in rat

Dr. Choudary stated, with general concurrence by the rest of the committee, that this tumor is not

usually considered when assessing relevant risk to humans. The following points were used to support this conclusion: pheochromocytomas have a high background rate in rats and in the NTP study, the increased incidences were only slightly higher than control. The occurrence of pheochromocytomas is often influenced by factors such as stress, and in the absence of significant increase in malignant tumors, may be considered to result from alterations in homeostasis when such conditions occur in the bioassay. Historically, the pheochromocytomas observed in this study would be dismissed as relevant to human risk based on the high background rate and the reported decrease in body weight, increase in renal toxicities, and non-tumor related mortality (suggests stress related effects).

Renal Tubule Adenomas in rat

The committee focused the discussion on the possibility that the renal adenomas reported in the NTP study were likely the result of excessive renal toxicity as supported by the increased nephropathy seen in rats. Moreover, the severity of the nephropathy was associated with tumor incidence. The NTP agreed that increases in nephropathy could lead to an increased incidence in renal tumors. However, they could not determine if toxicity induced nephropathy was the single contributing factor to the tumor incidence.

The committee also requested NTP to elaborate on the step-section analysis and its necessity. NTP indicated that this analysis was conducted to determine if there was a treatment effect in female rats. For completeness, the male rats were included in the analysis. A treatment effect was not found in the females, although the analysis confirmed the original renal tumor findings in the males.

Histiocytic Sarcomas in mouse

The committee raised some concern with the findings for this tumor type regarding its pathologic classification. The NTP stated that histiocytic sarcomas are easily distinguishable from malignant lymphomas. It was further contended that the expert pathologists reviewing the slides agreed with the histiocytic sarcomas differentiation from the other hemopoietic system tumors. It was noted that significant bone marrow suppression was observed in the mouse.

Malignant Lymphomas and Lymphomas of Thymic Origin

The committee again focused on the excessive dosing in the studies as the contributing factor leading to the reported bone marrow toxicities. The committee further questioned the shape of the dose response for the malignant lymphomas and was concerned by the reduced response at the high dose and a statistically significant response only in females. Although the NTP representatives stated that the increased toxicities could have resulted in a lower incidence of malignant lymphomas for the HD groups of both males and females, it was found to be statistically significant for all dose levels in the female mouse. A preliminary study is underway at NIEHS to improve the understanding of these tumor types and their origins. The main concern was for the lymphomas of thymic origin classification given their, generally, rare occurrence.

Ovarian Tumors

The committee questioned the NTP regarding the possible effect/response of different organs to the estrogenic/antiestrogenic activity of phenolphthalein. It was noted that this drug has estrogenic and antiestrogenic activities in rodents. Dr. Choudary questioned whether the observed non-malignant tumors were glandular hyperplasias - NTP did not support this contention. The NTP could not address this question until additional data on the estrogenic and antiestrogenic effects of phenolphthalein were available. At the present time, the NTP does not believe the ovarian tumors were induced by an estrogenic effect of the drug.

To summarize the discussion and voting, the CAC members relied upon their experience with previous products, the findings presented for phenolphthalein by the NTP and the accompanying reports, and the available additional studies conducted by the NTP and industry to address specific questions regarding comparative metabolism and genotoxicity. Not all issues supportive or against a risk for human use of phenolphthalein were able to be resolved by the data available, and it is noted that important studies including the further investigation of genotoxicity, estrogenic effects, and carcinogenic response in transgenic mice at lower doses are currently pending. These studies may provide further assurance. Given the information available at the time of the vote, the labeled use of phenolphthalein for short, intermittent periods, the committee, in general, expressed the following.

The tumors observed in the rat study do not appear to indicate a risk for use of phenolphthalein for humans. It was felt that the tumors were observed in association with high exposures and toxicity. Renal tumors occurred only in males in association with significantly increased severity of renal toxicity. The pheochromocytomas observed in males and females were almost exclusively benign, are tumors with a high spontaneous rate of occurrence, are often influenced by alterations in homeostasis (observed in this study as non-tumor related deaths, renal toxicity and lower body weights) and occurred at lower than the control incidence in high dose females. Historically, when occurring under such circumstances, we have dismissed pheochromocytomas as relevant to human risk.

The committee was split in their evaluation of the mouse tumor findings as evidence of significant human risk. Taken into consideration were the high exposure, uncertain evidence of genotoxicity relevant to humans, and presence of tumors primarily in an organ system that was a site of toxicity in mouse. The histiocytic sarcomas, which were increased in males and females, were not significantly increased at the low dose which resulted in a >50-fold systemic exposure than is achieved in humans. The increases in malignant lymphomas in males (not statistically significant) and females (statistically significant across all dose groups) was largely attributable to the increases in lymphomas of the thymic origin. Malignant lymphomas, in general, are common tumors in this strain of mice and occur with high frequency; in the absence of the thymic lymphomas (and their contribution to the overall lymphoma rate), there would be little evidence for an increased rate, and would not likely be cause for concern. The thymic lymphomas are considered rare, were increased in males and females, and were felt by several committee members to indicate a possible risk for humans. The stromal tumors were also considered by some to be evidence of human risk, although it was noted that these tumors may have been

associated with estrogenic and antiestrogenic activities, and were limited to the female mouse (no evidence of response in female rats).

Some of the committee members who indicated there was some risk for humans, would likely consider the overall risk minimal, given the high exposures, if there were clear evidence for an absence of genotoxicity under conditions relevant to human use. Such information may be derived from the pending studies of phenolphthalein. However, as evidenced in the vote, a small majority of members felt that, given the labeled short term use of phenolphthalein, human risk was insignificant based on the currently available data. In general, it is noted that the conclusions of CDER's Carcinogenicity Assessment Committee do not invalidate nor contradict the conclusions of the NTP and their advisors regarding the evidence that phenolphthalein causes tumor formation in rodents under the conditions examined in the bioassays conducted.

Committee Questions

1. **Do the carcinogenicity studies as conducted provide a valid assessment of the carcinogenic potential of phenolphthalein?**

   Yes - 12* No - 1

   *Although valid to assess carcinogenic potential, several committee members voting yes believed that the doses were excessive based on toxicities observed or on saturation of exposure across dose levels.

   If not, provide reasons. Doses in both studies exceeded MTD.

2. **Do the studies provide evidence of trans-species tumorigenic potential for phenolphthalein?** (The committee chair elaborated on the intent of this question, indicating that the question was designed to address whether or not specific tumor types were of concern for cross-species risk. Specifically, do those tumors seen in rodents provide evidence of tumor potential in human?)

   For rat? Yes - 5 No - 8

   For mouse? Yes - 9 No - 4

   If so which tumor types are of concern? For those committee members who voted yes for the rat, tumor multiplicity was stated as a concern not necessarily the specific tumor types. Committee members generally expressed a greater concern for the observed mouse tumors as having a potential to cross species.

   For the Rat? Renal adenomas - 4
   Pheochromocytomas - 1

For the Mouse? Thymic lymphomas - 9
Histiocytic sarcomas - 7
Sex-cord stromal - 3

If not, why? For those committee members who voted no, the general consensus was that tumors were probably related to high dose induced toxicities. The renal adenomas were generally dismissed based on the extent of renal toxicities observed, limitation to males and lack of evidence of renal toxicity in human. The pheochromocytomas were believed likely to be related to animal stress as discussed above. Moreover, these tumors, which have a high background rate in rats, have traditionally played little role in risk assessments when the increases are essentially limited to benign tumors.

3. **Do the studies addressing genotoxic potential and comparative metabolism and exposure provide information of potential relevance to human risk?**

Yes - 8 No - 3 Adequate data not provided - 1

a. **Do you conclude phenolphthalein is a likely genotoxin:**

For rodent?

Yes - 8 No - 3 Adequate data not provided - 1 Abstained - 1

If yes, why? Four of the 8 committee members voting in the affirmative indicated that the genotoxic results may be attributed to either a high-dose phenomena or an unusual study design with repeated dosing, and better data addressing these points would be useful.

If no, why? Committee members believe that phenolphthalein is not likely to be genotoxic because the evidence provided was insufficient to conclude the drug was a genotoxicant. Findings were limited to evidence of clastogenic activity which could have been associated with the toxicity observed.

For human?

Yes - 1 No - 5 Adequate data not provided - 6* Abstained - 1

*Committee members believed that lower doses should be examined to address the genotoxic potential in humans. In addition, the studies currently underway at NTP and those recommended by the CAC may provide adequate information to fully address the genotoxic potential of phenolphthalein.

b. **Is the metabolism of phenolphthalein and systemic exposure to**

phenolphthalein under the conditions of the bioassay in rodent sufficiently similar to that in humans under the conditions of standard use?

Metabolism? Yes - 7 No - 6

Committee members voting no indicated that insufficient data was provided to assess the similarities in metabolism under the conditions relevant to the bioassay. Because of the high exposures achieved in the bioassay compared to the concentrations tested in the in vitro studies, differences in metabolism could have occurred.

Systemic Exposure? Yes - 3 No - 8 Abstained - 2

The committee members voting no did not believe the rodent bioassays to be sufficiently similar to conditions of human use because the rodent exposure was excessive (including the lower doses) relevant to human exposure under normal use, making extrapolation to low human exposures difficult.

4. **Do you conclude that based on the tumor findings in the bioassay, the genotoxicity results and comparative metabolism and exposure information that the studies conducted provide evidence for carcinogenic potential of phenolphthalein relevant to human use?**

Yes - 5 No - 7 Abstained - 1

Please delineate your reasons (or refer to responses above).

Yes - The carcinogenic potential of phenolphthalein relevant human use is low but real if phenolphthalein is a genotoxic substance. The genotoxicity needs to be more fully assessed to draw conclusions on human relevance, or irrelevance.

No - The reported tumors were likely related to toxicity and high-dose exposures used in the studies.

The likelihood that phenolphthalein is genotoxic is low.

The carcinogenic potential does not appear relevant to humans for the labeled use. However, some relevance of these findings may apply for chronic misuse of phenolphthalein products.

5. **Although, the OTC division will make a decision based on the information currently available are there any specific studies which you believe should be conducted to further clarify this issue.**

Yes - 9 No - 4

Additional studies suggested:

1. Human epidemiologic study.
2. Assessment of saturation of metabolism/detoxification in relation to genotoxicity findings.
3. More complete exposure and metabolism data under the same conditions as the carcinogenicity studies.
4. Mouse lymphoma assay for point mutations.
5. Human cell transformation study.
6. DNA adducting study over a range of exposures.
7. P53 transgenic mouse study.

Joseph DeGeorge, Ph.D.
Acting Chair, CAC

ATTACHMENT I

Carcinogenicity Assessment Committee
April 2, 1996
Attendees

CAC Members
Joseph DeGeorge, Acting Chair, HFD-150*
Albert DeFelice, HFD-110
Charles Resnick, HFD-110
Norman See, HFD-540
Lucy Jean, HFD-170
Robert Osterberg, HFD-520
Jasti Choudary, HFD-180*
Ron Steigerwalt, HFD-510
William Fairweather, HFD-710
James Farrelly, HFD-530
Laraine Meyers, HFD-160
Virgil Whitehurst, HFD-570
Conrad Chen, HFD-550
Sharon Olmstead, Exec Sec, HFD-006

FDA Staff
Seung Hee Park, HFD-180
Robert Eshelman, HFD-312
Cheryl Turner, HFD-560
Mary Jane Walling, HFD-105
Indra Antonipillai, HFD-180
Jerry Young, HFD-180
Tanveer Ahmad, HFD-180
Yash Chopna, HFD-180
Kathleen Haberny, HFD-001
Michael Weintraub, HFD-105
Debra Bowen, HFD-560*
Barbara Hill, HFD-540
Anne Mustafa, HFD-560
Helen Cothran, HFD-560
Jason Brodsky, HFI-60
Mary Robinson, HFD-560

Industry Representatives
W. O'Donnell, Sandoz
E. Polasek, Pharmacia & Upjohn
George Latyszonek, Johnson & Johnson
John Tomaszewski, Bayer Consumer Care
Christopher D'Aleo, Warner-Lambert
Fred Huser, Sandoz
Michael Perry, Sandoz
Russ Hume, Sandoz
James Mangold, Sandoz
Francis Tse, Sandoz
Mark Gelbert, Sandoz*
Daniel Lapadula, Sandoz*
Rick Morrissey, Schering-Plough
John Clayton, Schering-Plough
David Brusick, Hazleton
Karen MacKenzie, Sandoz
Lorna Totman, NDMA
Christine Babiuk, Sandoz
Chris Celeste, AAC
Robert Mathews, Keller & Heckman

NTP Representatives
John Bucher, NIEHS*
June Dunnick, NIEHS*
Richard Hailey, NIEHS*

Trade Press Representatives
Susan Easton, "Tan Sheet"
Elizabeth Hinkle, "Washington Drug Letter"

*Presenters

APPENDIX A

NTP Levels of Evidence for Carcinogenic Activity

The levels of evidence for carcinogenic activity in the phenolphthalein study were based on NTP definitions which are:

Clear evidence of carcinogenic demonstrated by studies that are interpreted as showing a dose-related (i) increase of malignant neoplasms, (ii) increase of a combination of malignant and benign neoplasms, or (iii) marked increase of benign neoplasms if there is an indication from this or other studies of the ability of such tumors to progress to malignancy.

Some evidence of carcinogenic activity as demonstrated by studies that are interpreted as showing a chemical-related increase incidence of neoplasms (malignant, benign, or combined) in which the strength of the response is less than that required for clear evidence.

Equivocal evidence of carcinogenic activity as demonstrated by studies that are interpreted as showing a marginal increase of neoplasms that may be chemical related (uncertain findings, i.e.,, not certain that the effect is related to chemical treatment).

No evidence of carcinogenic activity is demonstrated by studies that are interpreted as showing no chemical-related increased in malignant or benign neoplasms.

The following journal articles were included as part of Annex C of F.J.C. Roe's comments. Due to copyright infringement laws we cannot display them. We have listed the citations for your information.

National Toxicology Program
Report on Carcinogens Group

Hodgkinson A, Davis D, Fourman J, Roberston WG, Roe FJC. 1982. A comparison of the effects of lactose and of two chemically modified waxy maize starches on mineral metabolism in the rat. Fd Chem Toxic 20:371-382.

Kemp CJ, Donehower LA, Bradley A, Balmain. 1993. Reduction of p53 gene dosage does not increase initiation or promotion but enhances makignant progression of chemically induced skin tumors. Cell 74:813-822.

Harvery M, McArthur MJ, Montogmery Jr CA, Butel JS, Bradley A, Donehower LA. 1993. Spontaneous and carcinogen-induced tumorigenesis in p53-deficient mice. Nature Genetics 5:225-229.